

Development of an 'In vitro'  
Neurotoxicity Assay

Michigan Univ.  
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Prepared for

Health Effects Research Lab.  
Research Triangle Park, NC

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| 16. ABSTRACT<br><p>The aim of this research project was to investigate the possibility of developing the neurotoxic esterase (NTE) assay into a totally <u>in vitro</u> system to enable neurotoxicity assessment to be carried out rapidly on large numbers of compounds. The purified exzyme was to be immobilized on a solid matrix for use in a continuous-flow reactor. Occurrence of NTE was confirmed for brain, spinal cord, and peripheral nerve of the hen. The enzyme was absent from most other tissues, with the notable exception of spleen, spleen lymphocytes, and circulating blood lymphocytes. Chick brain NTE appears to be identical to hen brain NTE with respect to a variety of characterization parameters, and is therefore recommended as a tissue source for future work on NTE. In both tissues, the greatest subcellular enrichment is in the microsomal (P<sub>3</sub>) fraction. NTE may be solubilized from hen or chick brain microsomes with retention of inhibitor characteristics of the native enzyme. Solubilized NTE is most stable when stored at -18°C in unbuffered Triton X-100 detergent solution. Immobilization of NTE was most successful using ionic binding to DEAE-Sephacel<sup>TM</sup>. However, this preparation was less stable than soluble enzyme stored under identical conditions.</p> |                                                  |                                                   |
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DEVELOPMENT OF AN IN VITRO NEUROTOXICITY ASSAY

by

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Under Grant No. R805339

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## FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The Laboratory contributes to a rational approach for regulating toxic substances through research programs aimed at increasing our understanding of adverse health effects and the development of better ways to predict toxicity. The work described in this report deals with the latter category--specifically, an enzyme test that can predict within a few hours a delayed neurotoxic response of organophosphorus compounds. This response had formerly required several weeks of clinical or histopathological evaluation to detect.

F.G. Hueter, Ph. D.  
Director  
Health Effects Research Laboratory

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## ABSTRACT

The aim of this project was to investigate the development of a totally in vitro neurotoxicity assay system using the enzyme neurotoxic esterase. Such a system was sought to allow rapid assessment of a large number of compounds for neurotoxicity.

In addition to a thorough biochemical characterization of several tissues containing neurotoxic esterase, a phylogenetic study of NTE occurrence was conducted. This search was made to identify sources of NTE for study.

Purified or partially purified neurotoxic esterase was to be immobilized on a solid matrix for eventual use in a continuous flow reactor.

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## SECTION 1

### INTRODUCTION

It is now known that some organophosphorus (OP) compounds initially developed as pesticides due to their anticholinesterase activity can also produce a delayed neurotoxic effect resulting in distal degeneration of nerve axons and concomitant ataxia and paralysis, which can be irreversible. This effect may be called, organophosphorus distal axonopathy (OPDA), and can also result from exposure to OP compounds used industrially that have little or no anticholinesterase activity. The problem has been extensively reviewed and a specific enzymological test, the neurotoxic esterase (NTE) assay, has been developed for evaluating the neurotoxic potential of OP compounds (Johnson, 1975; Davis and Richardson, 1980a). Johnson's assay provided a vast improvement over the older clinical and histopathological methods, but many unanswered questions remained concerning the relationship of the enzyme to the development of neurotoxicity. Furthermore, to be more useful as a screening technique for quickly evaluating large numbers of compounds for neurotoxicity, it would be highly desirable to circumvent in vivo dosing in hens, as is now done, by isolating the enzyme in such a way as to allow repeated tests in a completely in vitro system.

The principle of the assay is differential sensitivity of esterases to neurotoxic and non-neurotoxic OP compounds. In this report, a compound is designated "neurotoxic" strictly on the basis of its ability to produce the syndrome of OPDA. Thus, paraoxon (diethyl p-nitrophenylphosphate) is "non-neurotoxic" because it does not cause OPDA, although it produces acute neurotoxicity due to its inhibition of acetylcholinesterase. NTE is measured as the difference in esterase activity towards phenyl valerate between tissue aliquots preincubated either with a non-neurotoxic inhibitor or a combination of a non-neurotoxic and a neurotoxic inhibitor.

Complete inhibitor titration curves were used as an expanded form of the NTE assay to rigorously search for activity among species and tissues in order to learn more about the distribution of the enzyme and identify the best source for solubilization and isolation. Techniques were then developed for solubilization of NTE and its characterization in order to compare derived preparations to native enzyme. Solubilized enzyme was then attached by various means to various solid supports in order to prepare an



immobilized NTE that could eventually be used in a continuous-flow enzyme reactor as the key element in a totally in vitro test system.

## SECTION 2

### CONCLUSIONS and RECOMMENDATIONS

Brain tissue from a wide variety of primitive and advanced species was found to possess apparent NTE activity, with the possible exception of the shark and skate, where mipafox (N,N'-diisopropyl fluorophosphonoamidate)  $I_{50}$ 's were shifted to values approximately 10-fold higher than found in hen brain. Chick and hen brain were found to have comparable activity and were identical by all characterization parameters used. It is recommended that chick brain be used as a tissue source for future work on isolation of pure NTE.

Subcellular fractionation of hen and chick brain showed that up to 70% of the NTE is found in the  $P_3$  ("microsomal") fraction. This fraction was enriched in 5'-nucleotidase, a plasma membrane marker, but the origin of the NTE-bearing membrane is still open to investigation. Preparation of a microsomal fraction should be regarded as a useful first step in making purified NTE.

A major new finding was the existence of NTE in lymphocytes in the hen, rat, and human and in platelets in rat and human. By all criteria used to characterize the enzymic activity, hen lymphocyte and brain NTE are identical. Moreover, the activity responds similarly after dosing with neurotoxic OP compounds, if measurements are carried out at 4 hr post dosing. At 24 hr, some compounds do not produce similar responses on the brain and lymphocyte enzymes. Lack of correlation at longer times may be due to the dynamics of the lymphocyte pool compared with the relatively stable situation in brain, but this question demands further research.

Biochemical characterization of NTE activity included complete inhibitor titration curves, determination of inhibitor  $I_{50}$  values, substrate hydrolysis ratios, thermal inactivation curves, and pH profiles. These parameters were used to assess similarity of NTE activity from different sources or after different treatments, such as solubilization or immobilization. Additional indirect characterization consisted of measurement of stability under various conditions of medium composition and temperature. These measurements also had obvious direct practical value in determining optimum conditions for storage and in assessing the relative merit of different NTE preparations for eventual use as a component in an in vitro test system.

Solubilization of NTE from brain tissue was successfully achieved. The best preparations proved to be from hen or chick brain using Triton X-100 detergent. Studies were done in a systematic manner, maintaining strict attention to detergent/protein ratios. It was also found that incorporation of a lipid mixture, asolectin, into the solubilization step prevented loss of activity, indicating that NTE is a lipid-dependent enzyme. Solubilized NTE retains all of the characteristics of the native enzyme. This is a noteworthy and encouraging result in terms of looking ahead to further work on isolation and use of NTE in an in vitro test system. It should also be noted that brain from larger species (ovine; bovine) confer no advantage over chicken brain as a starting material for solubilization. These species gave activity that was relatively less stable or that differed in inhibitor characteristics from native chicken brain NTE.

A considerable quantity of detailed information on stability of NTE under various conditions has been compiled in this study. In terms of the most promising preparation for further development of purification and an in vitro test system, the best conditions for storage appear to be Triton X-100 solubilized NTE from chicken brain microsomes, stored unbuffered at -18°C.

Immobilization of NTE was attempted on a variety of supports using a number of binding methods. The most extensive studies were carried out on ion-exchange resins, using ionic binding. Covalent attachment to matrices resulted in extensive inactivation of the enzyme. The optimum immobilization system to date is binding of Triton X-100 solubilized NTE from chicken brain to DEAE-Sephacel™. The bound enzyme retains the inhibitor characteristics of native enzyme. However, the immobilized enzyme is not as stable as the soluble form under identical storage conditions.

Overall, it is recommended that efforts be continued to develop an in vitro assay system based on NTE. Because of the lack of stability of immobilized forms of the enzyme, it may still be necessary to perform assays in the batch mode. Emphasis should be placed on obtaining pure NTE in order to obviate the differential character of the assay. Also, further attention should be directed at convenient ways to assess the aging reaction, which is the second element in addition to simple inhibition that is required in order to have neurotoxicity (Johnson, 1974).

### SECTION 3

#### GENERAL METHODOLOGY

##### EXPERIMENTAL ANIMALS

White Leghorn "spent" hens at least 1 year old weighing 1.5-2.8 kg were obtained initially from Singing Hills Egg Farm, Clinton, MI and later from Omega Chicks, Haslett, MI. They were housed up to 4 per stainless steel cage, 0.8 x 0.8 x 0.9 m, in a temperature-controlled room with a light-dark cycle of 12 hr each. Hens were given Purina Layena<sup>TM</sup> Poultry Feed and water ad libitum. They were acclimated to these conditions for 2 weeks prior to use in experiments.

White Leghorn chicks were purchased as day-old females from Napoleon Hatchery in Napoleon, OH. They were kept in groups of 25 on 1/2" wire mesh floors in the cages described above. During the first two weeks an infrared lamp was suspended from the top of the cage to provide the extra warmth required by the chicks. They were fed Purina Startena<sup>TM</sup> Chick Feed and water ad libitum. No acclimatization period was observed for the chicks.

##### PREPARATION OF HOMOGENATES

Hens and chicks were sacrificed by decapitation with a stainless steel guillotine or stainless steel culinary scissors, respectively. Brains were removed immediately after sacrifice or as soon as possible if blood were being collected. Skulls were opened with heavy surgical scissors, bone cutters, and forceps. Brains were removed to ice cold buffer with the aid of a spatula. Membranes, bone chips, clotted blood, and visible blood vessels were removed with forceps and scalpel. Brain stems were truncated near the foramen magnum. Homogenizations were done in 6.5 times the tissue weight, in mls, of standard buffer in a cold glass-teflon homogenizer with a chamber clearance of 0.10 to 0.15 mm. Tissues were homogenized for 15 strokes at 2300 RPM. The homogenates were rinsed into cold graduated mixing cylinders and diluted to 10% W/V with buffer.

##### NTE ASSAY

NTE activity was determined according to the methods of Johnson (1969a; 1977). The second method is a time-saving adaptation of the first in which, at the end of the assay, tissue is solubilized with detergent rather than being removed by centrifugation. Modification of the assay required for individual

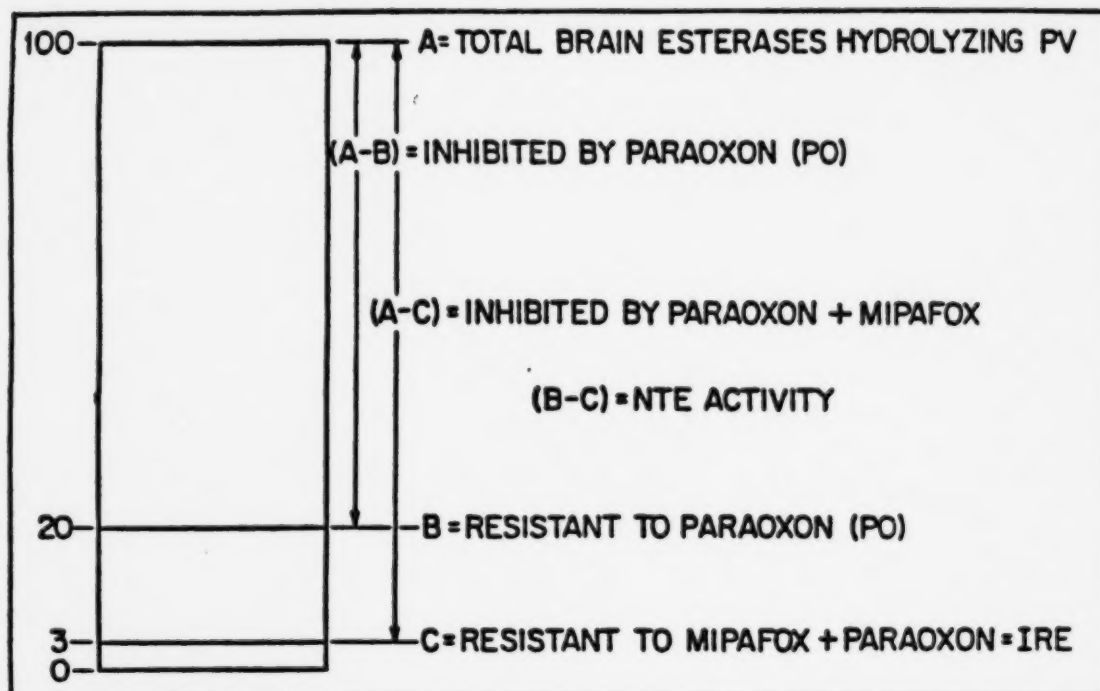


Figure 1. The schematic representation of NTE titration in hen brain homogenate.



experiments or use of tissues other than brain are described when the experiment is discussed. The second method was published with a printing error: the amount of tissue per tube should be 6.0 mg rather than 0.6 mg.

The neurotoxic esterase assay is based on the differential sensitivity of phenyl valerate (PV) hydrolyzing esterases to neurotoxic and non-neurotoxic inhibitors. In Figure 1 the total PV esterase activity in hen brain homogenate is designated by the quantity A, or, 100%, where no inhibitors are present. In condition B, paraoxon (PO), a non-neurotoxic inhibitor, has been preincubated with the brain homogenate. Paraoxon inhibits the quantity (A-B), about 80% of the total activity. Condition C represents preincubation with both paraoxon and the neurotoxic inhibitor mipafox. Addition of mipafox results in the inhibition of an additional 17%, the quantity (B-C). This quantity represents neurotoxic esterase (NTE) activity. Note that some residual activity still remains at high inhibitor concentrations: this activity is referred to as inhibitor-resistant activity (IRE). It is because of this residual activity that the assay must be done differentially.

The buffer used in the standard assay procedure is 50 mM Tris, 2 mM EDTA brought to pH 8.0 (25°C) with HCl. Throughout this report the terms "buffer" and "standard buffer" refer to this solution. In the standard two-point assay (Johnson, 1977), paired samples of tissue homogenate are preincubated in buffer at 37°C for 20 min with paraoxon (50 µM) plus either a) buffer or b) mipafox in a final volume of 2.0 ml. After preincubation, 2.0 ml of a phenyl valerate dispersion is added and the solution vortexed. Incubation is continued for 15 min. The dispersion of substrate is made by adding 31 volumes of 0.03% Triton X-100 (W/V) in H<sub>2</sub>O to 1 volume of 1.5% PV (W/V) in redistilled dimethylformamide and mixing thoroughly by swirling. The enzymatic reaction was stopped by adding 2.0 ml of 1% sodium dodecyl sulfate (W/V) in H<sub>2</sub>O containing 0.025% 4-aminoantipyrine (W/V) (SDS-AAP). Stock 4-aminoantipyrine is made up at 0.5% in 0.5 M Tris buffer pH 9.0 at 25°C (W/V). Color is developed by adding 1.0 ml of 0.4% K<sub>3</sub>Fe(CN)<sub>6</sub> in H<sub>2</sub>O (W/V) and is read at 510 nm. The molar extinction coefficient of the antipyrine dye reaction product is 13,900 under these conditions. NTE activity is represented by the difference in absorbance between conditions (a) and (b) respectively. Duplicate tubes are used for each point in a given single experiment. NTE and IRE activity is expressed as nmol phenol formed per min per specified quantity of enzyme source, usually per g tissue used, wet weight.

#### TITRATION CURVES

Paraoxon titration curves were generated by varying the paraoxon concentration in the preincubation medium over the concentration range 0.01 to 1000 µM. For tissues that showed very high phenyl valerate esterase activity, at low inhibitor concentrations, it was necessary to use an aliquot of the preincubation

mixture for incubation with phenyl valerate: i.e., all preincubations were done with identical amounts of tissue homogenate. For each tissue studied, inspection of the paraoxon titration curve, together with consideration of the absolute activity remaining at the higher paraoxon concentration yielded an optimum paraoxon concentration for use in the mipafox titration. At this paraoxon concentration, the (A-B) esterase activity will be inhibited.

Mipafox titration curves were generated by including paraoxon in the preincubation medium, at the selected optimum concentration, and mipafox, over the concentration range 0.001 to 1024  $\mu$ M.

When working with lymphocytes isolated from spleen and blood the assay and reagent volumes were reduced by a factor of 20 to accommodate the small amounts of tissue available. For peripheral nerve and small quantities of brain tissue, assay and reagent volumes were reduced by a factor of two. For each tissue studied, at least three complete titration curves were generated using each inhibitor.

#### SECTION 4

##### PHYLOGENETIC STUDY OF THE OCCURRENCE OF NTE

NTE has been found in neural tissue from birds, fish, reptiles, amphibians, and mammals whose ancestors evolved in the last 400 million years (MY). Characterization of the enzyme included determination of specific activity, apparent activation energy, and inhibitor  $I_{50}$ 's for phenyl valerate hydrolysis. The following species, whose ancestors evolved at the approximate time indicated within parentheses (Waterman, 1971) were examined: sheep (40 MY), steer (40 MY), lizard (320 MY), newt (373 MY), skate (405 MY), shark (405 MY), and sea lamprey (460 MY). Apparent NTE activity was found in all species examined, with the possible exception of skate and shark, where the mipafox and di-iso-propylphosphorofluoridate (DFP)  $I_{50}$ 's were shifted to higher values.

Mipafox  $I_{50}$  values were in the range of 6 to 25  $\mu\text{M}$  for all species except the skate and shark, for which values of 64 and 80  $\mu\text{M}$ , respectively, were found (Table I). NTE from the skate and shark also had higher  $I_{50}$ 's for DFP (5.3 and 6.2  $\mu\text{M}$ ) than NTE from newt, trout, lamprey, and chick (0.7 to 1.5  $\mu\text{M}$ ). The activities were distributed throughout the range from 2716 (chick) to 117 (lamprey) nmol/min/g tissue (wet weight). Energy of activation fell between 6.8 and 10.5 kcal/mole except for the shark (51 kcal/mole). This latter value needs to be redetermined. There is little correlation between these parameters and phylogenetic age, and no evolutionary pattern is discernable in the species examined to date. This protein appears to have originated in species whose ancestors arose earlier than 460 MY ago.

TABLE 1. PHYLOGENETIC STUDY OF THE OCCURRENCE OF NTE

| ANIMAL          | AGE<br>(MY) | ACTIVITY         |                  | I <sub>50</sub> <sup>c</sup> |         | ENERGY OF<br>ACTIVATION <sup>d</sup> |      |      |
|-----------------|-------------|------------------|------------------|------------------------------|---------|--------------------------------------|------|------|
|                 |             | NTE <sup>a</sup> | IRE <sup>a</sup> | Ache <sup>b</sup>            | Mipafox | DFP                                  | NTE  | IRE  |
| Sheep           | 40          | 1846             | 618              | 2.1                          | 9.1     | ---                                  | 7.3  | 0.9  |
| SE <sup>e</sup> |             | 268              | 114              | 0.2                          |         |                                      |      |      |
| Steer           | 40          | 1245             | 1125             | ---                          | 6.2     | ---                                  | ---  | ---  |
| SE              |             | 137              | 136              |                              |         |                                      |      |      |
| Rat             | 100         | 651              | 755              | 4.3                          | 13.1    | ---                                  | 8.4  | 2.2  |
| Chick           | 200         | 2716             | 685              | 8.4                          | 8.9     | 0.7                                  | 6.7  | 3.3  |
| SE              |             |                  |                  | 0.8                          |         | .01                                  |      |      |
| Hen             | 200         | 2426             | 702              | 6.3                          | 7.9     | 0.4                                  | 7.6  | 5.5  |
| SE              |             | 104              | 6                | 0.3                          |         |                                      |      |      |
| Trout           | 220         | 403              | 238              | ---                          | 11.5    | 1.5                                  | 7.6  | 0.9  |
| Lizard          | 320         | 343              | 599              | ---                          | 8.3     | ---                                  | 7.0  | 4.9  |
| Newton          | 373         | 178              | 145              | 4.6                          | 13.5    | 1.3                                  | 7.2  | 1.4  |
| SE              |             | 14               | 26               | 0.7                          | 0.7     | 0.1                                  |      |      |
| Shark           | 405         | 378              | 939              | 2.6                          | 79.5    | 6.2                                  | 51.0 | -9.7 |
| Skate           | 405         | 197              | 411              | ---                          | 63.5    | 5.3                                  | 10.4 | 4.0  |
| SE              |             | 36               | 64               |                              | 3.9     | 0.1                                  | 0.02 | 0.01 |
| Lamprey         | 460         | 117              | 162              | ---                          | 21.8    | 1.5                                  | 10.4 | 0.7  |

<sup>a</sup> nmol/min/g tissue.<sup>b</sup>  $\mu$ mol/min/g tissue.<sup>c</sup>  $\mu$ Molar.<sup>d</sup> kcal/mole.<sup>e</sup> Standard error. N = 3 when SE is given, except that N = 31 for hen NTE and IRE.



## SECTION 5

### DISTRIBUTION OF NTE IN TISSUES OF THE HEN; OCCURRENCE OF NTE IN BLOOD COMPONENTS OF THE RAT AND HUMAN

Prior to this study, the presence of NTE had been established by differential titration only in hen brain, although its existence in spinal cord and peripheral nerve had been inferred from a two-point assay. In the present study, complete paraoxon and mipafox titration curves were done in triplicate for the following tissues of the hen: brain, spinal cord, peripheral nerve, red muscle, white muscle, heart, liver, kidney, spleen, and lymphocytes isolated from spleen and blood. All tissues except lymphocytes were prepared as 10% homogenates in the standard 50 mM Tris, 2 mM EDTA buffer at pH 8.0.

#### METHODS

Brain homogenates were prepared as described in SECTION 3. Spinal cord and peripheral nerves were dissected out in a cold room at 4°C because of the amount of time required for these operations. The vertebral column was split open with bone shears; the cord removed, and the meninges peeled off with forceps. To obtain peripheral nerve, the ischiatic nerve, made up of the adherent tibial and fibular nerves, was located and freed from blood vessels and muscles beginning at the spinal cord and extending to approximately the level of the first leg joint. The collagenous epineurium of the isolated nerve was split with iris scissors, and the nerves separated. Iris scissors were again used to split the perineurium. The endoneurium, which contains Schwann cells, axons and connective tissue, was teased free using forceps, and minced (100 strokes) with small scissors. The mince was homogenized for 30-35 strokes; after every 5 strokes the homogenate was cooled in an ice bath. Finally the homogenate was passed through a nylon screen (330  $\mu$ m) stretched over the end of a truncated 10 ml plastic syringe to collect bits of enveloping sheaths which had not been removed in the isolation procedure. The screen was weighed before and after filtration to determine the weight of the residual tissue.

The gastrocnemius muscle (red muscle) was removed bilaterally. The collagenous sheath covering the muscle was peeled off and the tissue minced by 100 strokes of a small scissors. The mince was put through a tissue press and homogenized. The homogenate was then passed through a 333  $\mu$ m nylon screen to remove



residual collagenous material. A portion of the pectoralis muscle (white muscle) was removed from either side of the sternum and processed as described above.

Heart, kidney, liver, and spleen were freed of capsules and connective tissue were present, and minced (100 strokes) before homogenization.

Leukocytes were isolated from spleen and blood using Ficoll-Paque<sup>TM</sup> (Pharmacia Fine Chemicals, Inc.) according to the procedure in the accompanying technical booklet (Anonymous, 1975) and discussed by Böyum (1968). Spleens were removed to and minced in 15 ml room temperature balanced salt solution of the following composition: 5  $\mu$ M  $\text{CaCl}_2$ , 98  $\mu$ M  $\text{MgCl}_2$ , 540  $\mu$ M  $\text{KCl}$ , 140  $\mu$ M Tris, 130 mM  $\text{NaCl}$  and 0.01% anhydrous D-glucose, pH 7.6. The mince was stirred with a glass rod to free lymphocytes from the tissue and then strained through a double layer of sterile gauze into a siliconized beaker. The tissue was stirred in a second 15 ml aliquot of balanced salt solution and strained as before. The combined cloudy filtrate was centrifuged for 10 min at 600 x g<sub>avg</sub>. The pelleted leukocytes were resuspended in 4 ml balanced salt solution, layered onto 3 ml Ficoll-Paque<sup>TM</sup>, and processed as described in the references. The entire procedure was carried out at room temperature to prevent clumping of the white cells.

Blood was collected in heparinized test tubes from decapitated chickens, using a wide mouth funnel covered with a single layer of sterile gauze. Rat blood was collected from the inferior vena cava of anesthetized animals using a 26 gauge 1 1/2" needle and a 10 ml heparinized syringe. Human blood was obtained from an arm vein using a heparinized 15 ml Vacutainer<sup>TM</sup> and a 21 gauge needle. Blood from all three sources was processed at room temperature according to the instruction for Ficoll-Paque<sup>TM</sup>. The composition of the final preparation was checked by Wright's stain and light microscopy

#### TISSUE DISTRIBUTION IN THE HEN

Figures 2A and B through 13A and B show the phenyl valerate titration curves for the tissues studied: each point is the mean  $\pm$  SE of at least three experiments. Figures 2A and B are the differential titration curves for brain homogenate. They serve to illustrate the experimental design and define the criteria used for identification of NTE in brain and other tissues. In Figure 2A, the upper curve with solid circles connected by the continuous line represents the titration curve with paraoxon, over the concentration range  $10^{-8}$  to  $10^{-3}$ M. Phenyl valerate hydrolysis was extremely sensitive to paraoxon up to about 10  $\mu$ M. This part of the curve apparently corresponds to titration of two esterases (Poulsen and Aldridge, 1964). Inspection of this curve shows that paraoxon in the range of 10-100  $\mu$ M preincubated with the brain homogenate would completely inhibit these enzymes. The phenyl valerate esterase activity remaining corresponds to

the B activity in Figure 1. The dashed line connecting the open symbols in Figure 2 is the titration, with mipafox, of the residual activity remaining at a constant paraoxon concentration of 100  $\mu$ M. The decrease in activity caused by mipafox corresponds to (B-C) or NTE activity in Figure 1. This activity is equal to 2426 nmol phenyl valerate hydrolyzed/min/g wet weight tissue. Using paraoxon, the  $I_{50}$  for NTE, i.e., the concentration of paraoxon necessary in the preincubation medium to inhibit 50% of the NTE activity, was determined to be greater than 400  $\mu$ M. For a more detailed examination, the same mipafox titration curve is shown on an expanded scale in Figure 2B. Here, 100% corresponds to the residual activity remaining at the chosen paraoxon concentration before addition of mipafox. The decrease in activity upon mipafox addition to the preincubation medium is due to the inhibition of NTE. Using mipafox, the  $I_{50}$  of NTE is 7-8  $\mu$ M.

From these curves it is clear that there is a significant differential sensitivity to mipafox over the concentration range 1-100  $\mu$ M over and above any sensitivity to paraoxon alone. This demonstrates and confirms the presence of NTE in hen brain.

The differential sensitivity shown by brain homogenate in Figure 2A, i.e. the hydrolysis activity represented by the area in between the two curves over the concentration range 1-100  $\mu$ M, is the basis of a "two-point assay" for NTE in this tissue. This simplified assay is the standard procedure used in the screening of organophosphorus compounds for delayed neurotoxic potential in hens (Johnson, 1977).

If no differential sensitivity had been demonstrated, that is, if the mipafox titration curve had been indistinguishable from the paraoxon curve in Figure 2A, resulting in a "flat response" in Figure 2B, then NTE would have been considered not to be present. Taken together, these findings, in brain, define a set of criteria for NTE identification in the other tissues studied. By definition, NTE activity was phenyl valerate esterase activity that was resistant to paraoxon, while at the same time being sensitive to mipafox. By analogy to brain, any paraoxon-resistant, mipafox-sensitive activity should show considerable sensitivity to mipafox, i.e.  $I_{50}$  = 7-8  $\mu$ M and much greater resistance to paraoxon,  $I_{50}$   $\geq$  400  $\mu$ M.

Figures 3A and B are the titration curves for spinal cord. They show the occurrence of NTE activity corresponding to 23% of brain NTE activity.

Figures 4A and B indicate an absence of NTE in peripheral nerve: a finding contrary to published reports (Johnson, 1975). Peripheral nerve shows a low total phenyl valerate esterase activity - approximately 10% that of brain NTE activity (approximately 14,000 nmol phenyl valerate hydrolyzed/min/g wet weight for brain versus approximately 1,400 nmol/min/g wet weight for peripheral nerve). This implies that NTE, if present, would be represented by a very low amount of phenyl valerate hydrolysis.

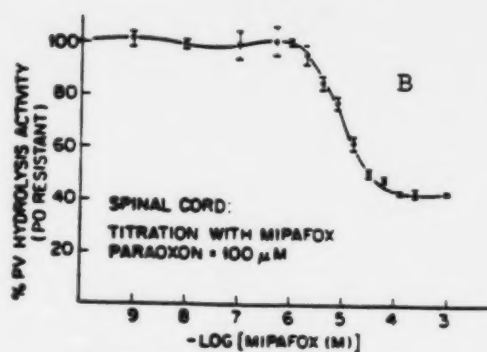
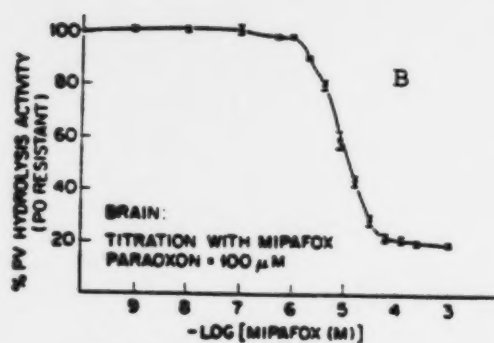
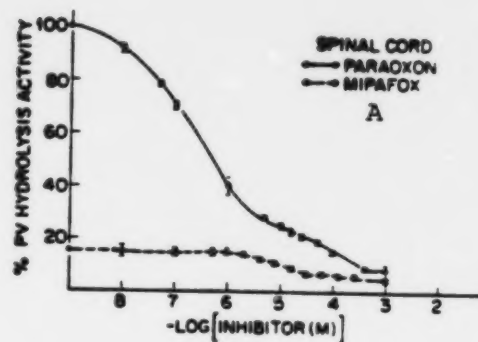
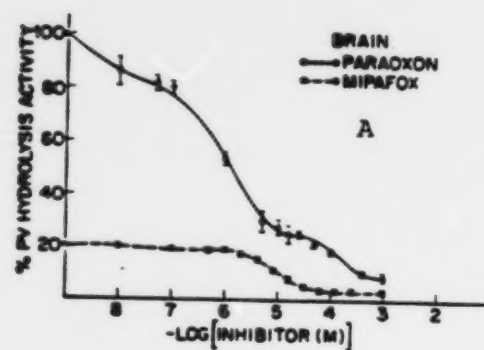


Figure 2A and B.

Figure 3A and B.

Figure 2A and B. Titration curves for hen brain phenyl valerate esterase activity.

Figure 3A and B. Titration curves for hen spinal cord phenyl valerate esterase activity.

This realization, coupled with the strict non-identity of the curves in Figure 4A and the unusual response in Figure 4B, prompted further investigation. Three experiments were tried. The first related to the sensitivity of the assay itself: a two-point assay for NTE was done on serially diluted brain homogenates. Results showed that NTE assay could detect (B-C) activity down to approximately 25 nmol phenyl valerate hydrolyzed/min/g wet weight tissue. Secondly, since detailed information on methods of tissue preparation were lacking for the published finding of peripheral nerve NTE, it was crucial to know if any of the material removed from the nerve during tissue preparation, i.e., epineurium and perineurium, had paraoxon-resistant activity. Assaying these tissue components revealed a low level of esterase activity that was sensitive to paraoxon but completely insensitive to mipafox. The third experiment involved increasing the tissue concentration in the assay tubes. Due to the high lipid content of peripheral nerve, initial experiments were done to determine the maximum tissue concentration useable in the assay without causing the assay tubes to remain cloudy after final color development. Using the maximum concentration of tissue, the titration curves shown in Figure 5A and B were obtained. The presence of NTE is therefore confirmed in peripheral nerve at approximately 42 nmol phenyl valerate hydrolyzed/min/g wet weight tissue.

Figures 6A and B show the titration curves for red muscle phenyl valerate esterase activity: NTE was not found. It must be pointed out that the term red muscle, as used in this discussion, refers to the high-oxidative biochemistry and presence of red myoglobin in this tissue. Red muscle, in this context, does not indicate the type of fiber present in this tissue, i.e., fast or slow twitch. The particular red muscle assayed was the gastrocnemius. It is composed of approximately 40% fast fibers. Innervation in muscles, i.e., the number of motor end plates per fiber, is known to be inversely related to the speed of the muscle (Hess, 1961). This distinction according to number of end plates per fiber is important because there is evidence indicating that NTE is present in synaptosomes isolated from hen brain (Richardson *et al.*, 1979). Since the muscle assayed had a high proportion of fast fibers, i.e., a low proportion of neuromuscular junctions, and since the neuromuscular junction could be considered a correlate of the synaptosome, then the question of the presence of NTE in this muscle is still unresolved. However, using the simpler two-point assay, NTE activity was found in the anterior latissimus dorsi muscle of the hen. This muscle is composed exclusively of slow fibers (Gauthier *et al.*, 1978). Interestingly, the NTE activity of this muscle is close to that of peripheral nerve. These findings may point to a similar role for NTE at the neuromuscular junction and at the synaptic junction.

Figures 7A and B show the titration curves for white muscle. The muscle used was the pectoralis: a muscle composed largely of



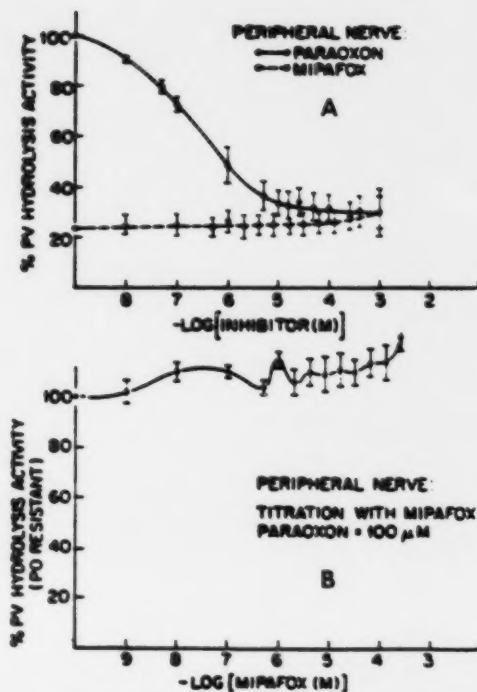


Figure 4A and B.

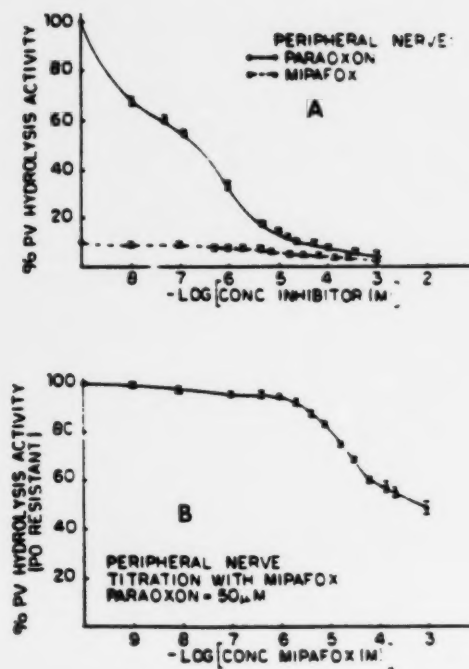


Figure 5A and B.

Figure 4A and B. Titration curves for hen peripheral nerve phenyl valerate esterase activity.

Figure 5A and B. Titration curves for hen peripheral nerve phenyl valerate esterase activity using more tissue.



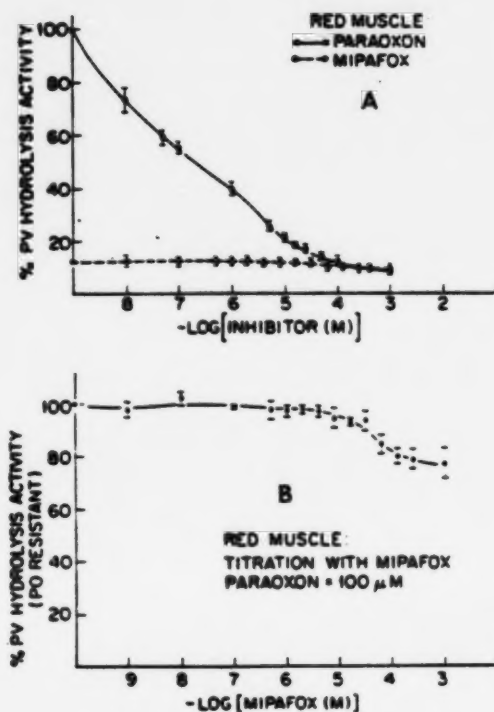


Figure 6A and B.

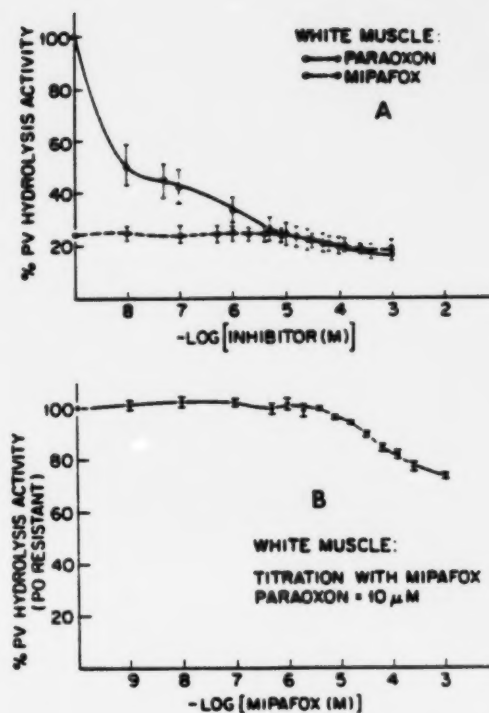


Figure 7A and B.

Figure 6A and B. Titration curves for hen red muscle phenyl valerate esterase activity.

Figure 7A and B. Titration curves for hen white muscle phenyl valerate esterase activity.

fast fibers. No NTE activity was found in white muscle -- a result in line with the hypothesis of few neuromuscular junctions in this tissue.

Figures 8A and B are the differential titration curves for heart. They demonstrate a differential sensitivity to mipafox with maximum sensitivity occurring over the same mipafox concentrations as in brain. However, the shape of the mipafox curve is qualitatively different from the corresponding curve for brain. Also, at high paraoxon concentration (1000  $\mu$ M), there was no differential sensitivity. Since the heart receives parasympathetic and sympathetic innervation, it has properties similar to those of skeletal muscle, including nerve endings. These nerve endings may be the source of the NTE activity demonstrated by differential titration. NTE activity is therefore tentatively identified in heart at 14% of that of brain.

Liver (Figures 9A and B) and kidney (Figures 10A and B) show no NTE activity using this method and criteria. Liver, however, had the highest total phenyl valerate esterase activity, i.e., A activity, of all tissues studied - approximately 340,000 nmol phenyl valerate hydrolyzed/min/g wet weight tissue.

Spleen in Figures 11A and B shows an obvious differential sensitivity to mipafox together with an unexplained stimulation in activity at low mipafox concentrations. The sensitivity to mipafox occurs over the same concentration range as in brain. There is therefore clear evidence of NTE activity in spleen. This activity is equal to approximately 70% that of brain NTE activity on a per gram wet weight basis.

The NTE activity in spleen suggested that activity could be present in spleen lymphocytes. Figures 12A and B show the titration curves for spleen lymphocytes. The curves show clearly that there is differential sensitivity to mipafox. The mipafox curve, Figure 12B, is quite similar to the brain curve and shows approximately the same  $I_{50}$  value. Using paraoxon the  $I_{50}$  for spleen lymphocyte NTE was greater than 400  $\mu$ M: the same as for brain NTE. Neurotoxic esterase is therefore present in spleen lymphocytes. On a per gram wet weight of tissue basis, this activity is approximately 26% that of brain.

Figures 13A and B show the differential titration curves for blood lymphocytes. There is obvious sensitivity toward mipafox. The curves are very similar to the spleen and spleen lymphocyte curves: the maximum mipafox sensitivity occurs over the same concentration range as in brain. Using paraoxon the  $I_{50}$  value is again similar to brain: greater than 400  $\mu$ M. Overall the lymphocyte curves bear a striking resemblance to the brain curves. NTE activity is therefore identified in blood lymphocytes. Red blood cells as well as plasma were found to have no paraoxon-resistant phenyl valerate esterase activity sensitive to mipafox.

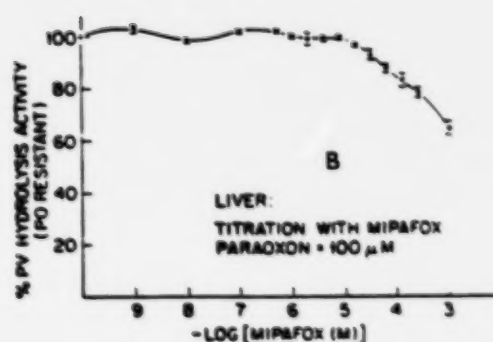
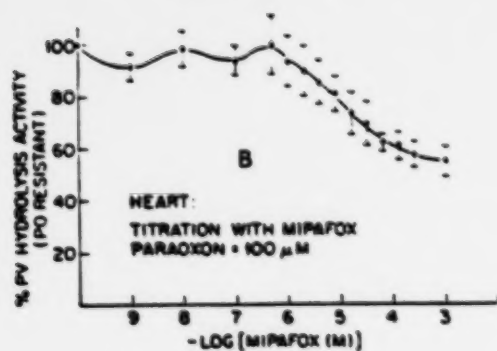
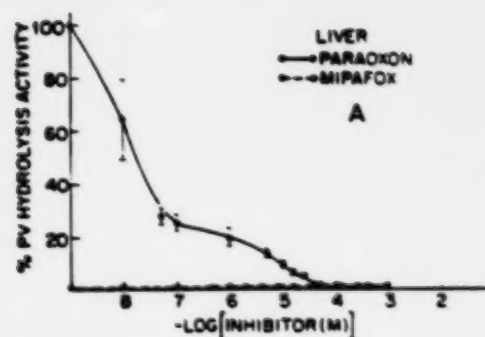
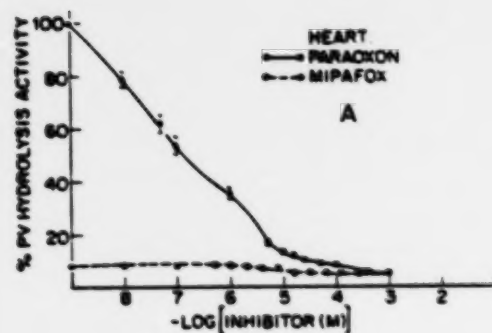


Figure 8A and B.

Figure 9A and B.

Figure 8A and B. Titration curves for hen heart phenyl valerate esterase activity.

Figure 9A and B. Titration curves for hen liver phenyl valerate esterase activity.

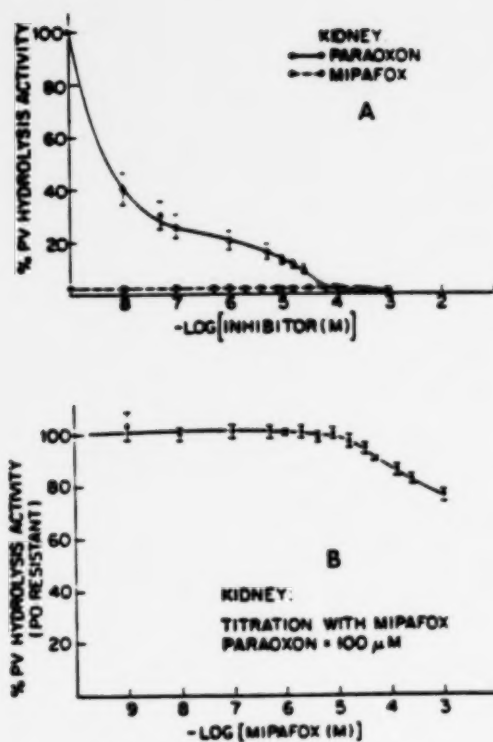


Figure 10A and B.

Figure 10A and B. Titration curves for hen kidney phenyl valerate esterase activity.

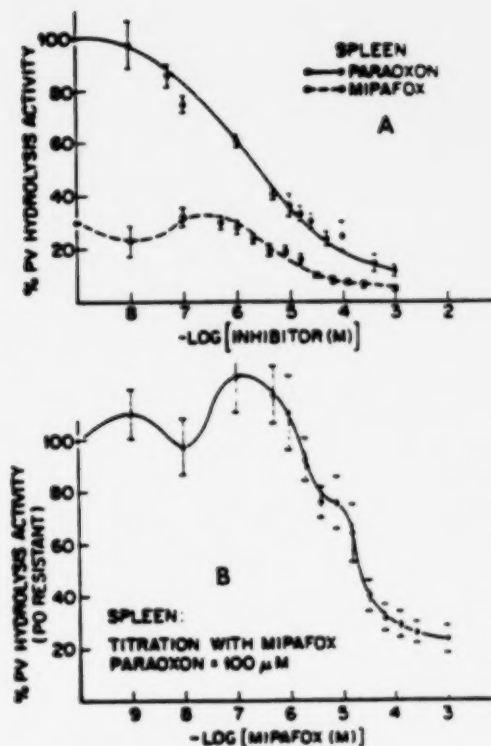


Figure 11A and B.

Figure 11A and B. Titration curves for hen spleen phenyl valerate esterase activity.

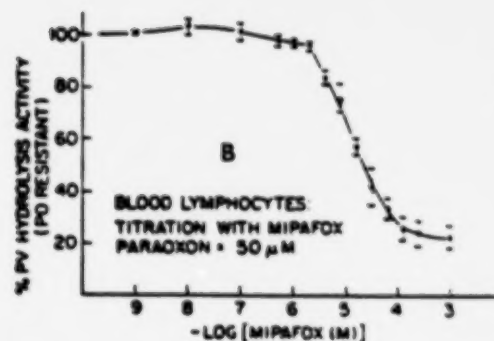
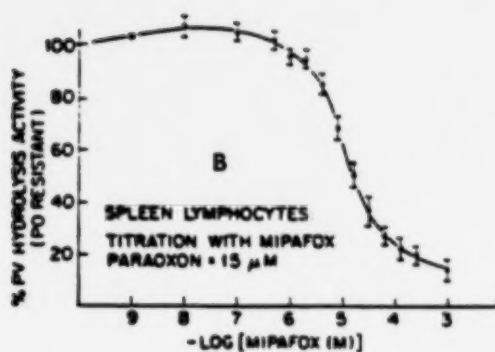
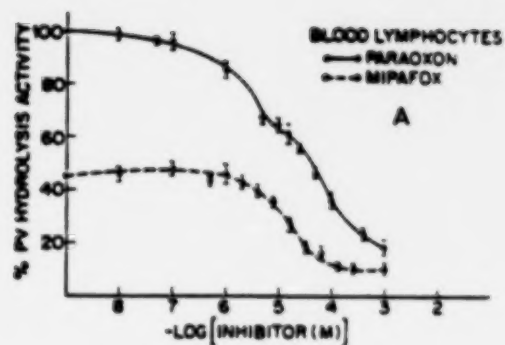
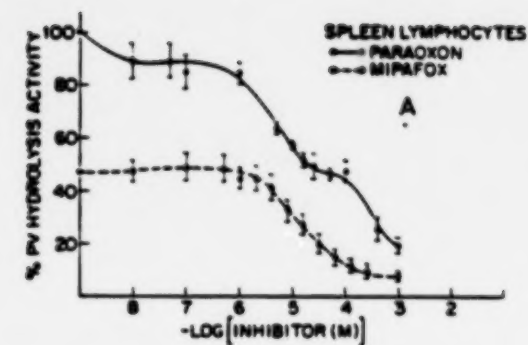


Figure 12A and B.

Figure 13A and B.

Figure 12A and B. Titration curves for hen spleen lymphocyte phenyl valerate esterase activity.

Figure 13A and B. Titration curves for hen blood lymphocyte phenyl valerate esterase activity.



Table 2 shows a summary of the tissue occurrence of NTE in the adult hen. Neurotoxic esterase activity is expressed in units of nmol phenyl valerate hydrolyzed/min/g wet weight tissue and in terms of percent of brain activity.

TABLE 2. NTE ACTIVITY IN TISSUES OF THE ADULT HEN

| TISSUE             | NTE ACTIVITY<br>(nmol/min/g<br>wet weight tissue) | % OF<br>BRAIN<br>NTE |
|--------------------|---------------------------------------------------|----------------------|
| Brain              | 2426.0 + 104.0                                    | 100.0                |
| Spinal Cord        | 510.3 + 45.7                                      | 21.0                 |
| Peripheral Nerve   | 42.0 + 2.1                                        | 1.7                  |
| Red Muscle         | 00.0                                              | 0.0                  |
| White Muscle       | 00.0                                              | 0.0                  |
| Heart              | 333.0 + 53.5                                      | 13.7                 |
| Liver              | 00.0                                              | 0.0                  |
| Kidney             | 00.0                                              | 0.0                  |
| Spleen             | 1686.0 + 24.0                                     | 60.5                 |
| Spleen Lymphocytes | 629.0 + 60.0                                      | 25.9                 |
| Blood Lymphocytes  | 592.5 + 42.3                                      | 24.4                 |

#### OCCURRENCE OF NTE IN RAT AND HUMAN LYMPHOCYTES AND PLATELETS

Figures 14-16 show differential titration curves for rat brain, lymphocytes, and platelets. The curves for human lymphocytes and platelets are given in Figures 17 and 18. These curves are very similar to those obtained for hen brain and lymphocytes (Fig. 2 and 13) and show the same  $PI_{50}$  for mipafox. Table 3 gives the NTE activity in nmol per mg protein assayed and the  $PI_{50}$  for these tissues. It is evident that rat brain has about half the NTE activity of hen brain. NTE is relatively plentiful in both lymphocytes and platelets of the human.

TABLE 3. NTE ACTIVITY IN BRAIN AND BLOOD COMPONENTS FOR HEN, RAT, AND HUMAN

| SPECIES | TISSUE      | $PI_{50}$ | NTE ACTIVITY <sup>a</sup> |
|---------|-------------|-----------|---------------------------|
| Hen     | Brain       | 5.10      | 23.1 + 0.8 <sup>b</sup>   |
|         | Lymphocytes | 4.95      | 11.0 + 0.6 <sup>b</sup>   |
| Rat     | Brain       | 5.06      | 9.8 <sup>c</sup>          |
|         | Lymphocytes | 5.02      | 8.5 <sup>c</sup>          |
|         | Platelets   | 5.04      | 9.2 <sup>c</sup>          |
| Human   | Lymphocytes | 5.06      | 17.0 + 1.2 <sup>d</sup>   |
|         | Platelets   | 5.05      | 13.2 + 0.2 <sup>d</sup>   |

<sup>a</sup>nmol/min/mg protein.

<sup>b</sup>Mean + SE, n = 22.

<sup>c</sup>Single determination.

<sup>d</sup>Mean + SE, n = 3.

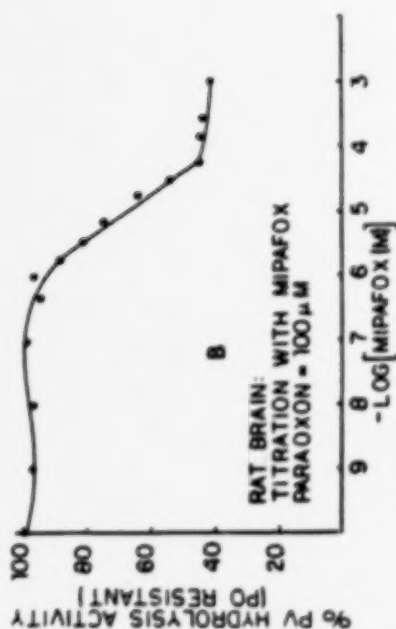
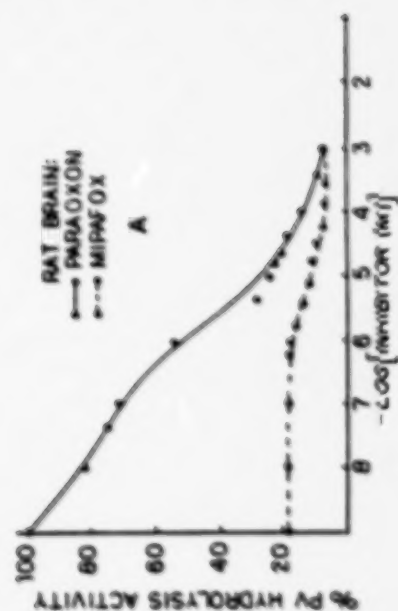


Figure 14A and B.

Figure 14A and B. Titration curves for rat brain phenyl valerate esterase activity.

Figure 15A and B. Titration curves for rat lymphocyte phenyl valerate esterase activity.

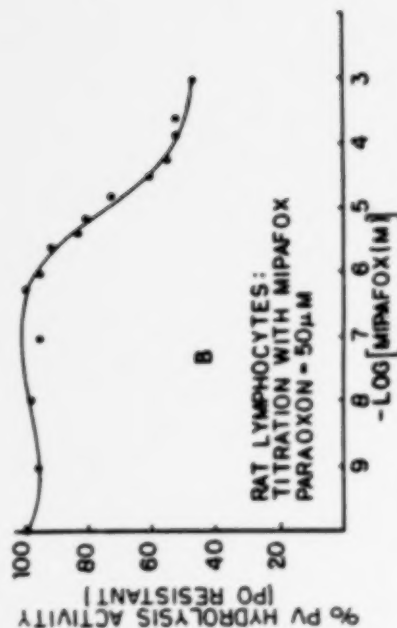
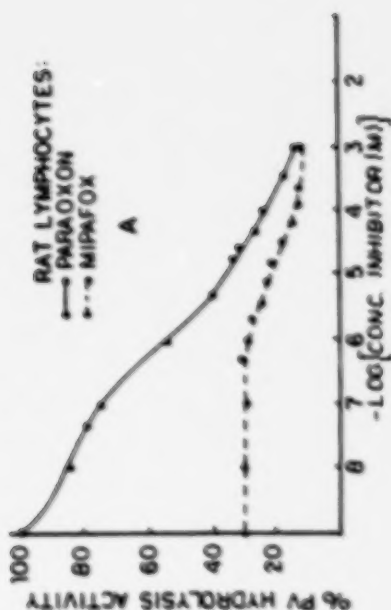


Figure 15A and B.

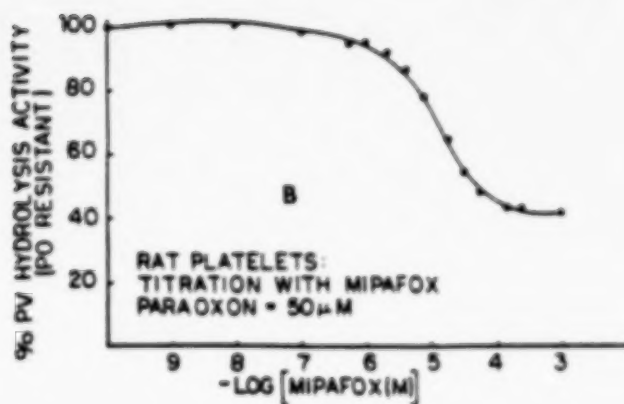
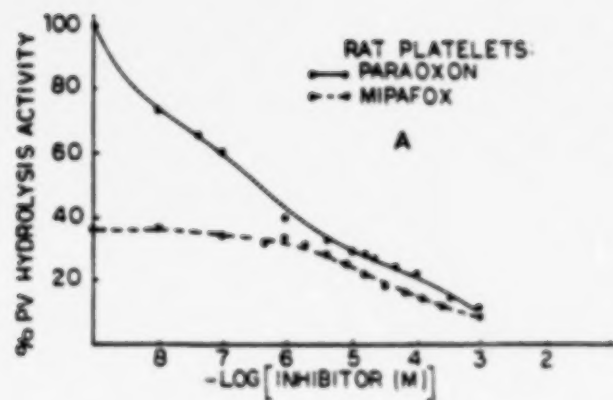


Figure 16A and B. Titration curves for rat platelet phenyl valerate esterase activity.

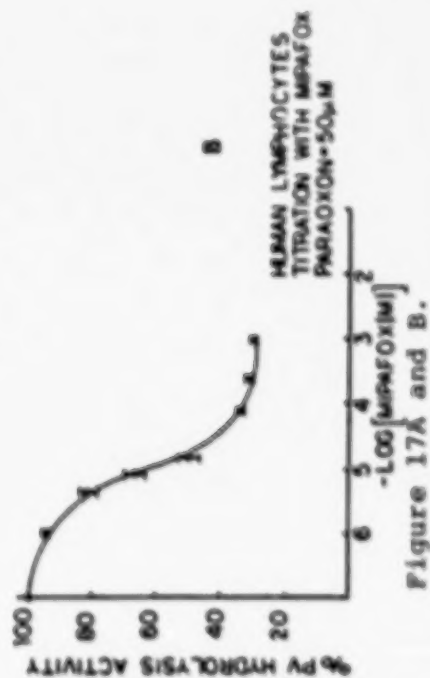
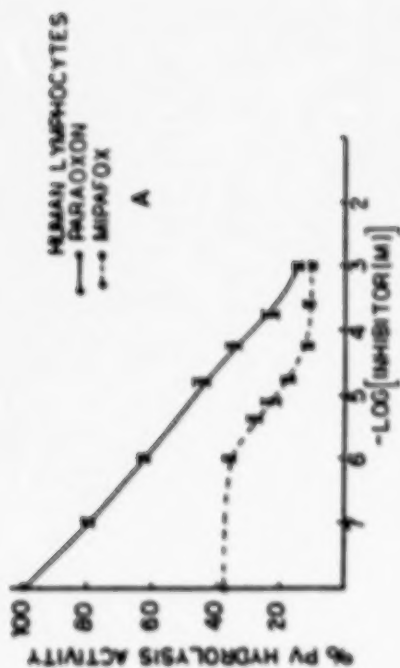


Figure 17A and B.

Figure 17A and B. Titration curves for human lymphocyte phenyl valerate esterase activity.

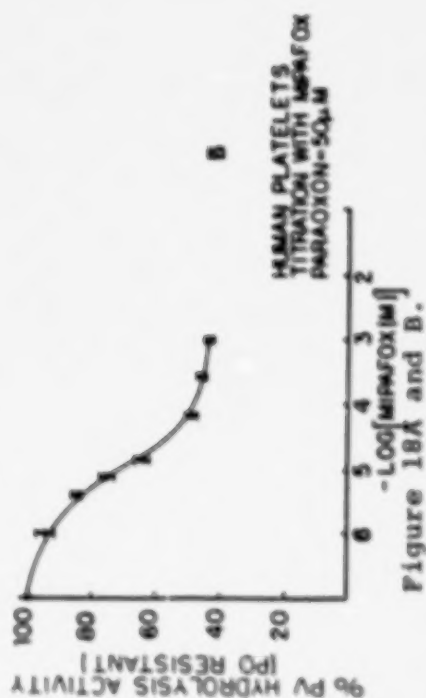
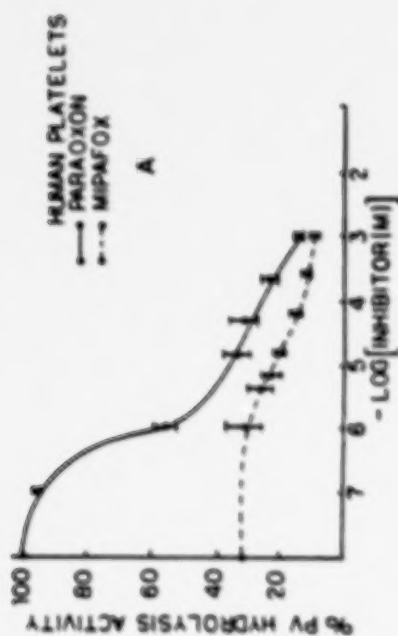


Figure 18A and B.

Figure 18A and B. Titration curves for human platelet phenyl valerate esterase activity.

## SECTION 6

### A COMPARISON OF BIOCHEMICAL CHARACTERISTICS OF BRAIN, LYMPHOCYTE, AND PLATELET NTE

The presence in hen lymphocytes of a phenyl valerate hydro-lase yielding a mipafox titration curve very similar to that obtained for NTE in brain (SECTION 5) suggests that these enzymes might be identical. If they are and if the enzyme from the two sources responds in the same way to neuropathic organophosphorus compounds (OPs), then lymphocyte NTE activity might be usable as a biomonitor for OP exposure. Moreover, any peripheral blood component which contains NTE might serve.

Some of the enzymological characteristics used to establish the properties of enzymes are: heat denaturation, pH-activity relationship, substrate specificity, and inhibitor specificity. Lymphocyte, platelet, and brain phenyl valerate hydrolases from hen, rat, and human were compared with respect to these characteristics. A correlation between hen lymphocyte and brain NTE activity after dosing with neurotoxic and non-neurotoxic compounds is reported in SECTION 7.

#### TISSUE PREPARATION

Brain homogenate, lymphocytes, and platelets were prepared as described in SECTIONS 3 and 5. Lymphocytes were resuspended after the final wash to  $2.8 \times 10^7$  cells/ml in standard buffer for homogenization. Platelets were resuspended in enough buffer to give a degree of cloudiness similar to that of the lymphocyte resuspension.

#### HEAT DENATURATION

Preliminary experiments showed that 55°C was optimal for heat denaturation studies using hen NTE. For the rat enzyme, 50°C was used. Homogenates of brain and lymphocytes were incubated at these temperatures for periods of time between 1 and 90 min. The samples were then transferred to an ice bath and diluted with cold standard buffer to give a final tissue concentration of 6 mg/ml for brain and  $1.12 \times 10^7$  cells/ml for lymphocytes. Aliquots of these dilutions were assayed for NTE activity. Brain homogenate was also assayed for acetylcholinesterase according to the method of Ellman *et al.* (1961) except that Iso-OMPA (tetra-iso-propyl pyrophosphoramidate) was used to inhibit pseudo-cholin-



esterase in place of quinidine (Aldridge, 1953).

The heat denaturation curves obtained from these experiments are shown in Figures 19-21. Each point represents the mean + SE of three experiments. It is apparent that hen lymphocyte and brain NTE denature at similar rates under these conditions. Furthermore, acetylcholinesterase denatures at a different rate, indicating that NTE denaturation is not simply due to general denaturation of proteins at this temperature.

Initial studies revealed that rat NTE denatures very rapidly at 55°C. This is a significant difference from the hen enzyme. Since a similar increased heat sensitivity was not observed in the chick (Fig. 20), it appears that this characteristic is not related to the resistance of both species to OPDA, or that species and age resistance are due to distinct mechanisms. At 50°C, NTE in rat platelets and brain denature at approximately the same rate (Fig. 21).

#### INHIBITOR CHARACTERISTICS

The titration curves for hen brain and lymphocyte NTE using phenyl valerate as substrate and mipafox as inhibitor are given in Figure 22. Similar curves were obtained using phenyl phenylacetate (PPA) as substrate and DFP as inhibitor as well. The basic method of titration is given in SECTION 3. When PPA was used as substrate, the amount of tissue assayed was increased by a factor of 5 (Johnson, 1969a). DFP concentrations ranged from 0 to 102.4  $\mu$ M. From a plot of percent activity (ordinate) versus -log of the molar inhibitor concentration (abscissa), the value on the ordinate corresponding to 50% inhibition of NTE activity was calculated from the following equation:

$$\begin{aligned} \text{ordinate value} = & \frac{100\% \text{ activity at mipafox/DFP at } 1024 \mu\text{M} / 102.4 \mu\text{M}}{2} \\ & + \% \text{ activity at mipafox/DFP at } 1024 \mu\text{M} / 102.4 \mu\text{M} \end{aligned}$$

The  $pI_{50}$ , equal to -log concentration of inhibitor at 50% NTE inhibition was equal to the corresponding value on the abscissa.

Titration curves are shown in Figures 23-25. Each point is the mean + SE of three experiments. Table 4 shows the  $pI_{50}$ 's corresponding to the combination of inhibitor and substrate used for each source of NTE. For a given substrate,  $pI_{50}$  values for the two inhibitors are significantly different from each other. For different substrates,  $pI_{50}$  values for the same inhibitor are the same, regardless of tissue source. Large variations in this ratio would strongly suggest that the hydrolysis activity is due to more than one enzyme.

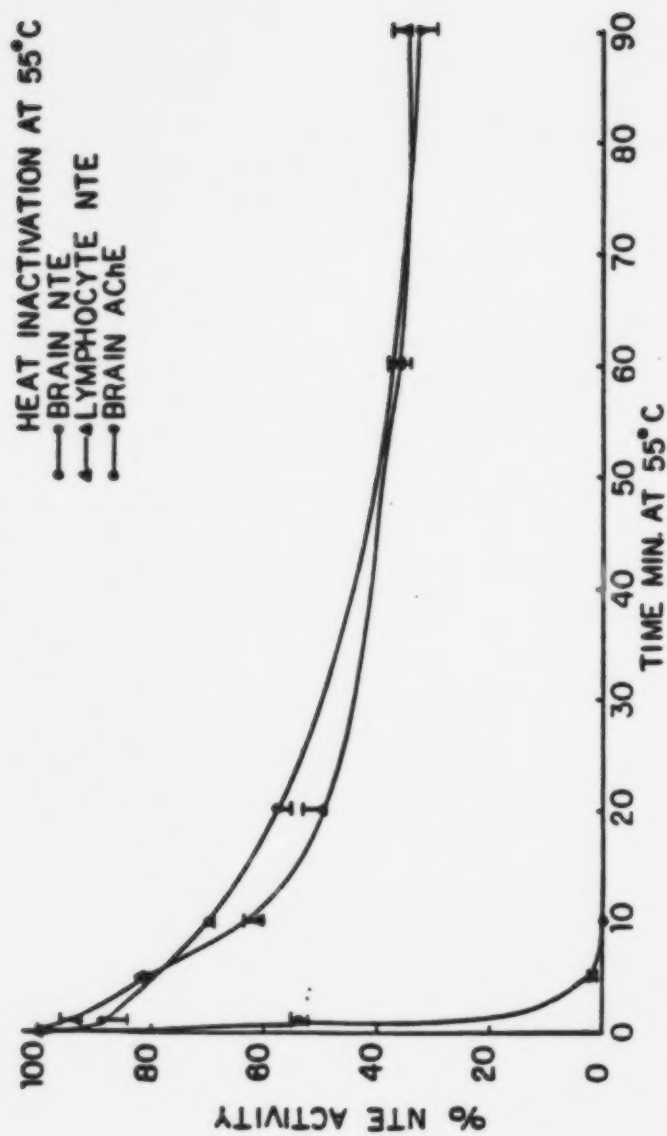


Figure 19. Heat inactivation at 55°C for NTE in hen brain and lymphocyte homogenates and acetylcholinesterase in hen brain homogenate. Points are mean  $\pm$  SE,  $n = 3$ .

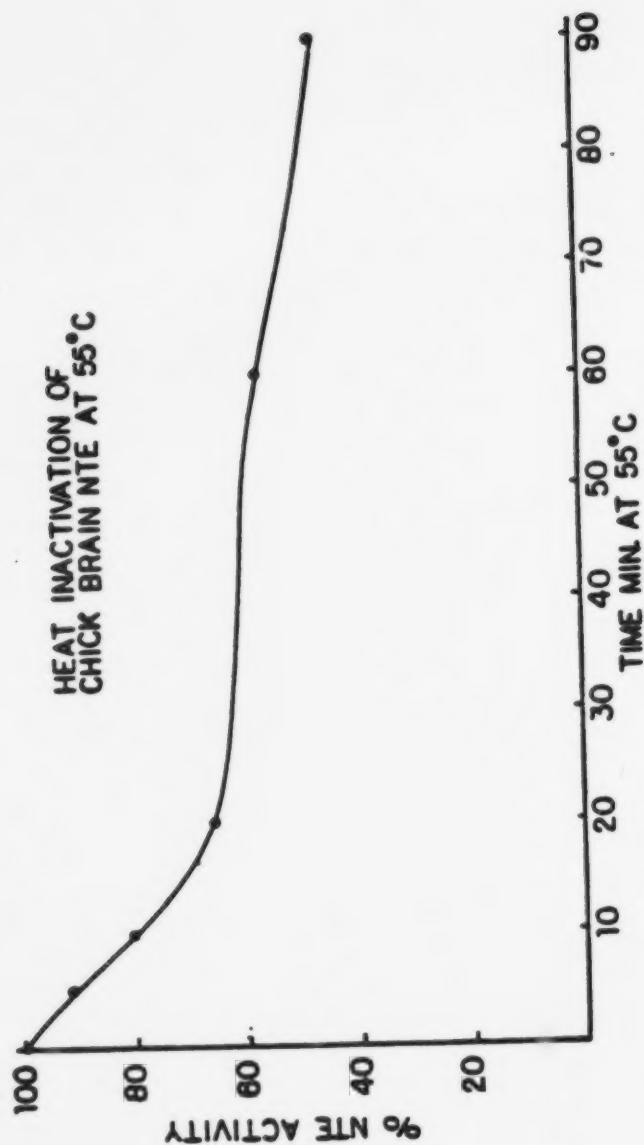


Figure 20. Heat inactivation at 55°C for NTE in chick brain homogenate. Points are from a single experiment.

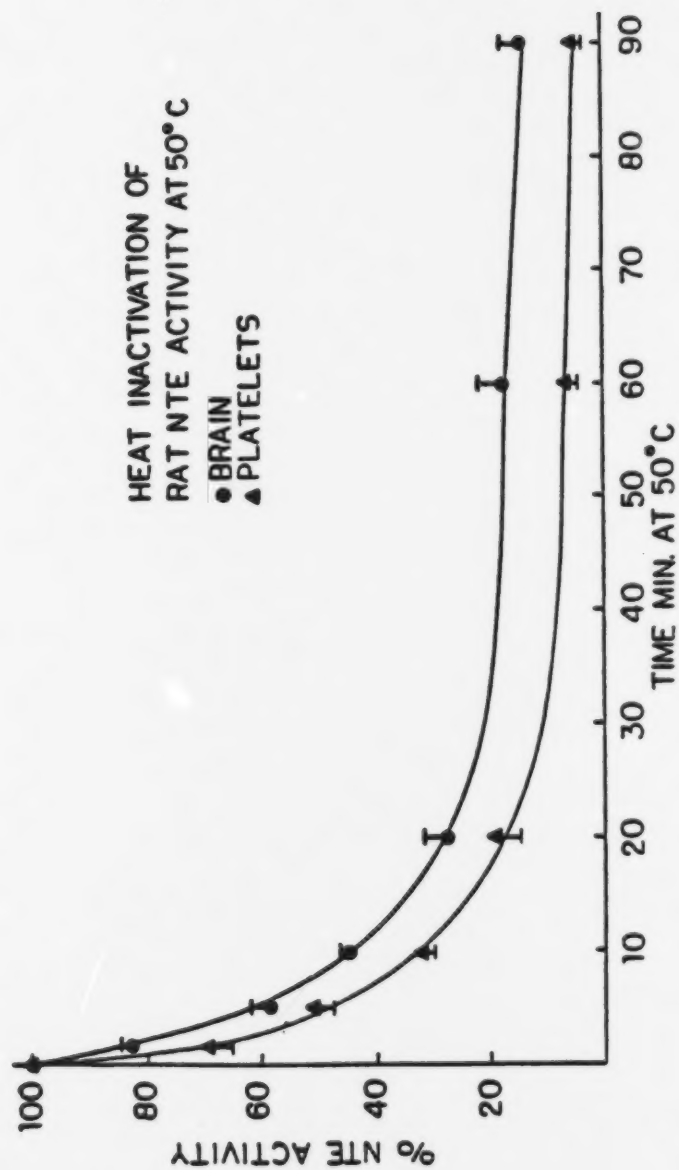


Figure 21. Heat inactivation at 50°C for NTE in rat brain and platelet homogenate. Points are mean  $\pm$  SE,  $n = 3$ .

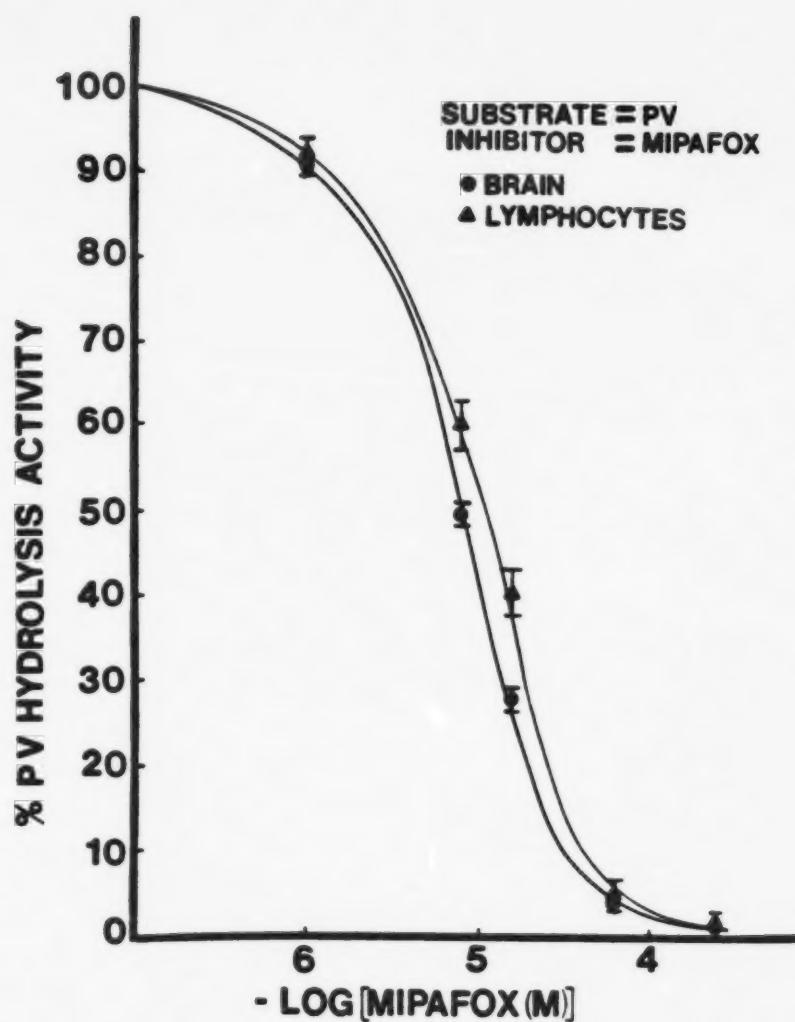


Figure 22. The titration of NTE in hen brain and lymphocyte homogenates using phenyl valerate (PV) as substrate and mipafox as inhibitor. Points are mean  $\pm$  SE,  $n = 3$ .



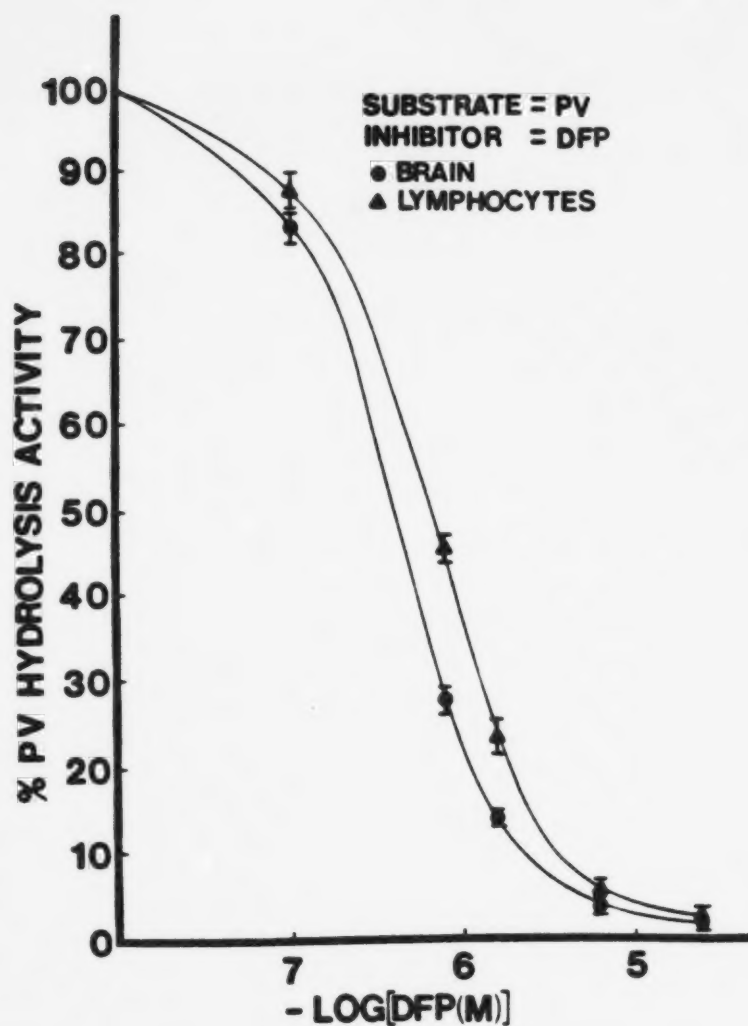


Figure 23. The titration of NTE in hen brain and lymphocyte homogenate using phenyl valerate (PV) as substrate and DFP as inhibitor. Points are mean  $\pm$  SE,  $n = 3$ .

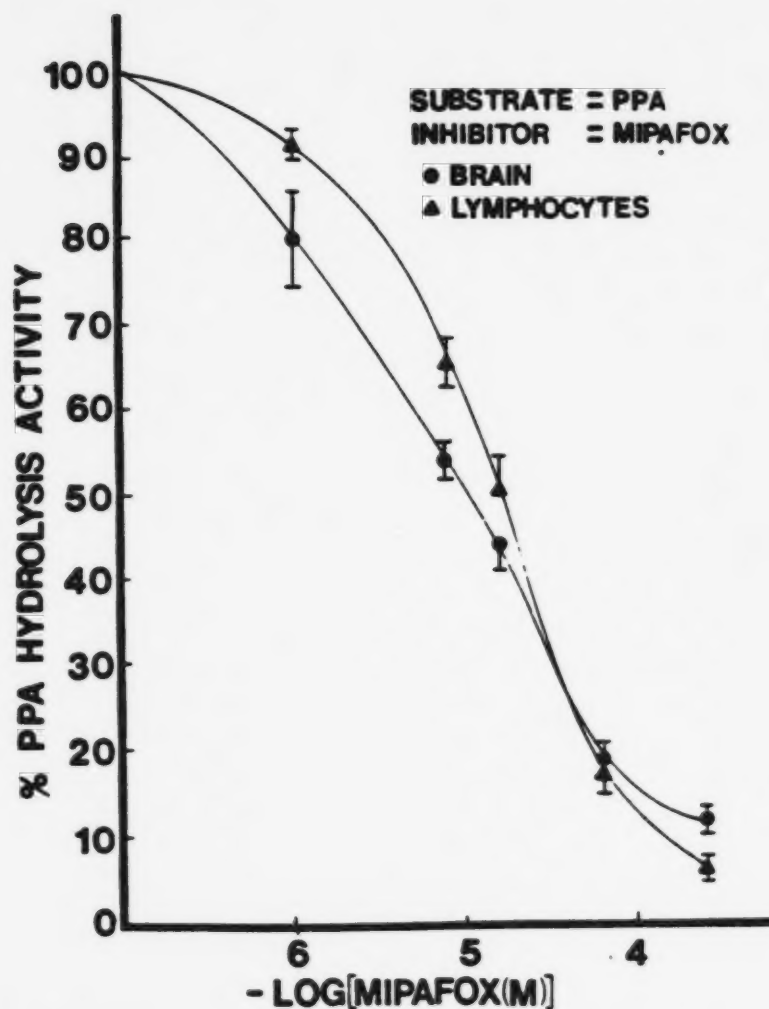


Figure 24. The titration of NTE in hen brain and lymphocyte homogenates using phenyl phenylacetate (PPA) as substrate and mipafox as inhibitor. Points are mean  $\pm$  SE,  $n = 3$ .

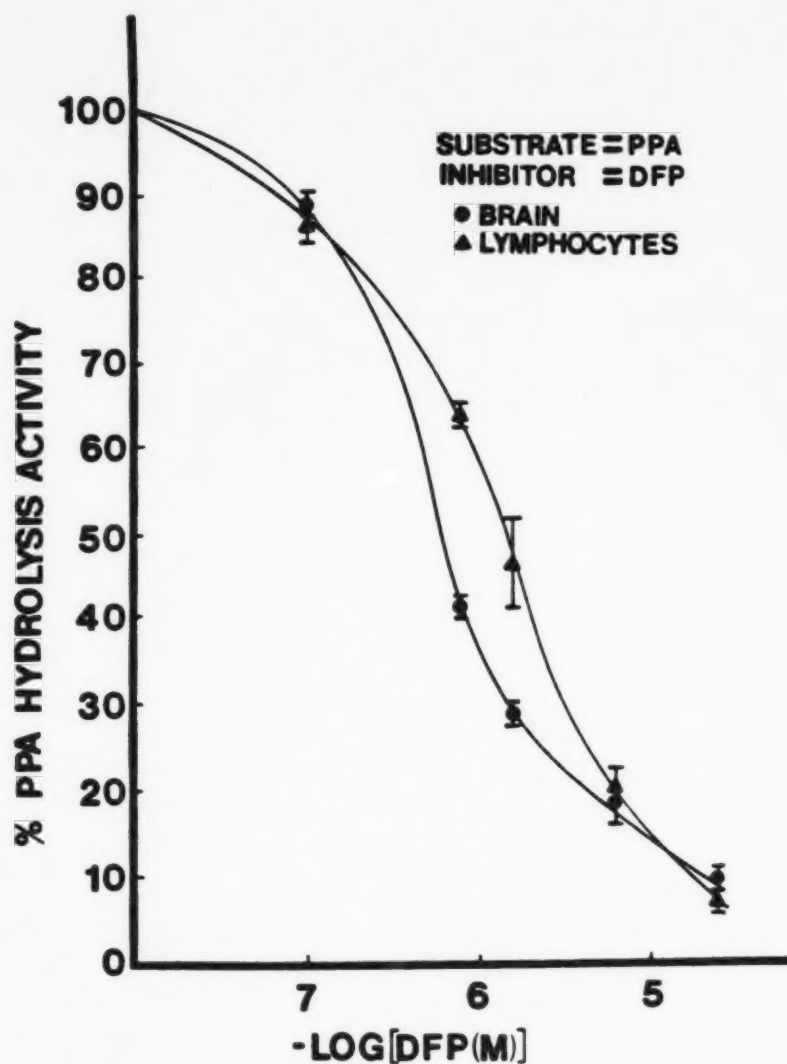


Figure 25. The titration of NTE in hen brain and lymphocyte homogenates using phenyl phenylacetate (PPA) as substrate and DFP as inhibitor. Points are mean  $\pm$  SE,  $n = 3$ .

TABLE 4. SUBSTRATE AND INHIBITOR SPECIFICITIES  
FOR NTE FROM TWO TISSUES OF THE HEN

| SUBSTRATE                     | INHIBITOR | NTE SOURCE  | PI <sub>50</sub> |
|-------------------------------|-----------|-------------|------------------|
| Phenyl Phenylacetate<br>(PPA) | Mipafox   | Brain       | 5.10             |
|                               |           | Lymphocytes | 4.90             |
|                               | DFP       | Brain       | 6.22             |
|                               |           | Lymphocytes | 5.96             |
| Phenyl Valerate<br>(PV)       | Mipafox   | Brain       | 5.10             |
|                               |           | Lymphocytes | 4.95             |
|                               | DFP       | Brain       | 6.42             |
|                               |           | Lymphocytes | 6.18             |

#### HYDROLYSIS RATIOS

Poulsen and Aldridge (1964) used hydrolysis ratios to show the presence in hen brain homogenates of at least two esterases that hydrolyze phenyl phenylacetate and phenyl 3-phenyl propionate. The method finds its greatest application for proving if the hydrolysis of two substrates, in unfractionated samples, is by a single enzyme. In this study, it is used to infer that the activity in two tissue homogenates is attributable to the same enzyme(s).

Tissues were prepared and NTE assayed exactly as described for the determination of the pI<sub>50</sub>'s except that the concentration of mipafox and DFP, in the preincubation tubes ranged from 0 to 20  $\mu$ M and 0 to 2  $\mu$ M respectively. The hydrolysis ratio, at a specific inhibitor concentration, was calculated by dividing the specific activity obtained for phenyl valerate by that obtained for phenyl phenylacetate. This ratio was plotted against mipafox or DFP concentration (Fig. 26). The ratios for each tissue at each inhibitor concentration are quite similar, indicating that the hydrolase activity in these tissues is attributable to the same enzyme. There does seem to be a shift in the ratio with increasing inhibitor concentration. This suggests that these substrates are hydrolyzed by more than one enzyme. A striking feature of the data is that these enzymes behave similarly in the two tissues with respect to the two inhibitors and, moreover, the ratio of substrate hydrolysis changes in the same way for both tissues.

#### pH-ACTIVITY RELATIONSHIPS

NTE from brain and blood components of hen, chick, and rat was compared with respect to pH-activity relationships. Before these experiments could be done, it was necessary to determine

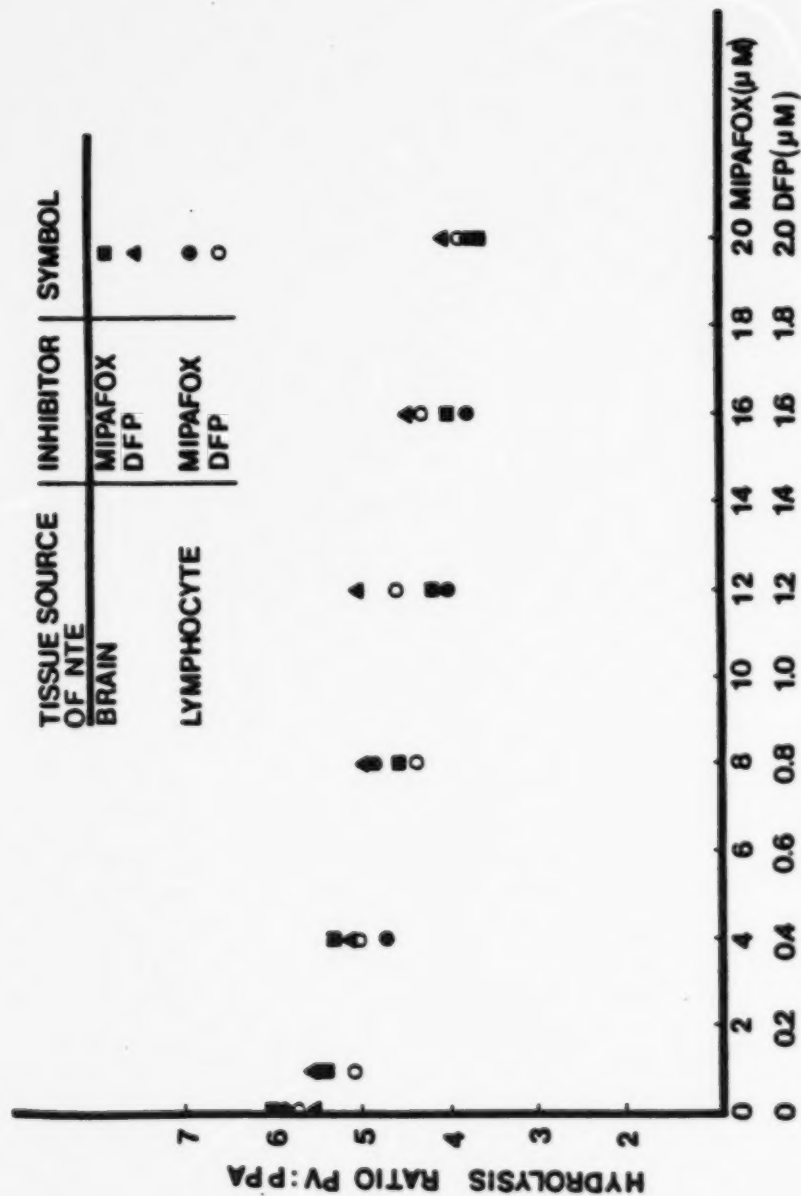


Figure 26. The influence of mipafox and DFP on the phenyl valerate (PV): phenyl phenylacetate (PPA) hydrolysis ratio for NTE in hen brain and lymphocyte homogenates.



the effect of pH on spontaneous hydrolysis of substrate and color development in the NTE assay.

The effect of pH on color development was studied over the range of 4 to 11 (Table 5). Four "Good" buffers and sodium acetate/acetic acid at 50 mM, without added EDTA, were used to achieve the various pH's (see table). A phenol standard made up in each of the buffers of interest was used in place of an enzyme preparation. The appropriate buffer of interest was substituted wherever Tris-EDTA, pH 8.0, is used in the standard assay, and phenyl valerate was omitted. Optimal color development was seen at pH 8.0 and 9.0.

TABLE 5. THE EFFECT OF pH ON COLOR DEVELOPMENT IN THE NTE ASSAY

| BUFFER           | pH*  | ABSORBANCE | % OF HIGHEST ABSORBANCE |
|------------------|------|------------|-------------------------|
| CAPS             | 11.0 | 0.494      | 79.4                    |
|                  | 10.5 | 0.562      | 90.4                    |
|                  | 10.0 | 0.593      | 95.3                    |
| BIS TRIS PROPANE | 9.5  | 0.602      | 96.8                    |
| TRIS             | 9.0  | 0.622      | 100.0                   |
|                  | 8.0  | 0.621      | 99.8                    |
|                  | 7.0  | 0.388      | 62.4                    |
| MES              | 6.0  | 0.021      | 3.4                     |
| Acetate          | 5.0  | 0.012      | 1.9                     |
|                  | 4.5  | 0.016      | 2.6                     |
|                  | 4.0  | 0.010      | 1.6                     |

\*Buffers made to this pH at 25°C.

The effect of pH on the spontaneous hydrolysis of phenyl valerate is shown in Table 6. In this experiment, phenyl valerate was added to 25 mM buffer of interest. After 15 min of incubation at 37°C, SDS-AAP in 100 mM Tris, pH 8.0, instead of the usual 50 mM Tris, was added. The lower concentration of buffer of interest and the more concentrated Tris-SDS-AAP were used so that while the pH during spontaneous hydrolysis would range from 4.0 to 11.0, the Tris-SDS-AAP would bring the pH back as close as possible to 8.0 for color development. The final pH of each sample was measured after the absorbance had been read, and is

given in the table. The spontaneous hydrolysis of phenyl valerate at 37°C is minimal at or below pH 8.0, but increases rapidly at higher pH's. Color development may not have been maximal in the last 4 or 5 samples, but it is not likely that the conclusions drawn would be affected.

TABLE 6. THE EFFECT OF pH ON THE SPONTANEOUS HYDROLYSIS OF PHENYL VALERATE

| BUFFER              | pH OF<br>HYDROLYSIS <sup>a</sup> | pH OF COLOR<br>DEVELOPMENT <sup>b</sup> | ABSORBANCE |
|---------------------|----------------------------------|-----------------------------------------|------------|
| CAPS                | 11.0                             | 8.32                                    | Off Scale  |
|                     | 10.5                             | 8.21                                    | 3.31       |
|                     | 10.0                             | 8.12                                    | 1.18       |
| BIS TRIS<br>PROPANE | 9.5                              | 8.28                                    | 0.718      |
| TRIS                | 9.0                              | 8.11                                    | 0.176      |
|                     | 8.0                              | 7.92                                    | 0.046      |
|                     | 7.0                              | 7.80                                    | 0.032      |
| MES                 | 6.0                              | 7.77                                    | 0.032      |
| Acetate             | 5.0                              | 7.88                                    | 0.023      |
|                     | 4.5                              | 7.73                                    | 0.034      |
|                     | 4.0                              | 7.60                                    | 0.034      |

<sup>a</sup> Buffer made to this pH at 25°C.

<sup>b</sup> Corrected to 25°C.

The effect of pH on phenyl valerate hydrolase activity was determined for brain NTE from adult hen, chick, and rat platelets and hen peripheral lymphocytes. These tissues were prepared as described in SECTIONS 3 and 5 except that the working enzyme suspension was made in 10 mM Tris, 2 mM EDTA, pH 8.0, instead of 50 mM Tris, 2 mM EDTA. Inhibitors were also made up in the more dilute buffer. After a 19.5 min incubation of the enzyme with inhibitors, the pH of the assay mixture was changed by the addition of 0.1 M buffer over the pH range of 4.0 to 9.0. KCl was added to these buffers before use to give a final ionic strength of 0.1 in assay tubes during substrate hydrolysis. In the presence of KCl, ionic strength differences between the buffers used to vary pH are assumed not to influence the reaction rate. Higher pH's were not used because the spontaneous hydrolysis of phenyl valerate were obscure any effects on enzymatic hydrolysis.

Substrate was added as usual at 20 min into the assay. The SDS-AAP solution used to stop the reaction was made up in 0.2 M Tris, pH 8.8, so that the pH of the assay mixture would be high enough to insure full color development.

The high concentration of  $K^+$  in the reaction mixture caused the SDS to precipitate (Maizel, 1971). Centrifugation at  $3000 \times g_{avg}$  for 15 min at room temperature gave a clear supernatant for absorbance readings.

The results of these experiments are given in Table 7 and in Figures 27 and 28. The values in the table represent the average of three experiments for all tissues except chick brain, for which the determination was done only once. The SE for each average was less than 5%. It is apparent that the effect of pH on NTE activity is similar for all 5 enzyme preparations under these conditions. All plots have peak activity between pH 6.5 and 7.0, and NTE from all sources tested was much more sensitive to pH on the acidic side compared to the alkaline side of neutrality.

Many esterases hydrolyzing phenol esters show a similar response (Bergman, 1958). This sensitivity on the acidic side is attributed to a catalytic imidazol group contained in histidine (Bruice and Gaston, 1956; Brestkin and Rozengart, 1965). The decreased pH sensitivity on the alkaline side could be due to weak catalysts, possible mediated by hydrogen bonding (Bergman et al., 1958), or by a group either with low alkaline pH sensitivity or with decreased catalytic activity at the high ionic strength of the hydrolysis medium.

Table 7 contains analogous information for IRE. In this case, activity maxima were seen from pH 6.5 to 8.5 for the 5 preparations. Overall, the curves obtained for IRE (Fig. 28) were less similar than those obtained for NTE. Since IRE is not the focus of study, these differences were not explored.

TABLE 7. THE EFFECT OF PH ON NTE AND IRE ACTIVITY AT HIGH IONIC STRENGTH

| BUFFER  | PH  | ENZYME SOURCE |       |             |       |           |      |                |      |              |       |     |     |
|---------|-----|---------------|-------|-------------|-------|-----------|------|----------------|------|--------------|-------|-----|-----|
|         |     | HEN BRAIN     |       | CHICK BRAIN |       | RAT BRAIN |      | HEN LYMPHOCYTE |      | RAT PLATELET |       | NTE | IRE |
|         |     | NTE           | IRE   | NTE         | IRE   | NTE       | IRE  | NTE            | IRE  | NTE          | IRE   |     |     |
| TRIS    | 9.0 | 77.7          | 56.7  | 82.6        | 94.2  | 66.0      | 73.9 | 67.6           | 97.0 | 76.0         | 62.5  |     |     |
|         | 8.5 | 79.7          | 74.7  | 77.7        | 92.2  | 65.7      | 83.7 | 68.6           | 99.8 | 70.5         | 63.3  |     |     |
|         | 8.0 | 80.4          | 85.8  | 81.5        | 100.0 | 71.6      | 93.3 | 72.4           | 94.1 | 67.8         | 78.2  |     |     |
|         | 7.5 | 84.6          | 100.0 | 88.9        | 97.4  | 76.7      | 95.1 | 77.9           | 82.6 | 76.4         | 89.4  |     |     |
|         | 7.0 | 97.9          | 80.4  | 99.6        | 70.6  | 85.2      | 96.3 | 91.4           | 67.8 | 81.1         | 95.4  |     |     |
| MES     | 6.5 | 93.8          | 86.8  | 100.0       | 78.6  | 97.0      | 92.3 | 100.0          | 65.8 | 99.1         | 100.0 |     |     |
|         | 6.0 | 85.8          | 61.6  | 85.7        | 51.3  | 92.7      | 61.7 | 86.5           | 51.4 | 90.5         | 94.0  |     |     |
|         | 5.5 | 76.7          | 47.2  | 75.5        | 53.8  | 80.6      | 37.0 | 80.5           | 47.8 | 75.0         | 77.7  |     |     |
| Acetate | 5.0 | 55.9          | 27.5  | 54.9        | 18.8  | 53.4      | 30.9 | 56.1           | 38.7 | 46.0         | 76.5  |     |     |
|         | 4.5 | 37.6          | 15.5  | 36.2        | 7.8   | 30.0      | 14.8 | 40.9           | 22.8 | 27.1         | 63.4  |     |     |
|         | 4.0 | 28.2          | 7.1   | 19.2        | 0.0   | 8.7       | 12.0 | 16.3           | 23.2 | 4.0          | 54.7  |     |     |

Table entries are % of maximal activity observed for each preparation, and are the average of 3 experiments for all samples except chick brain, which was done only once.

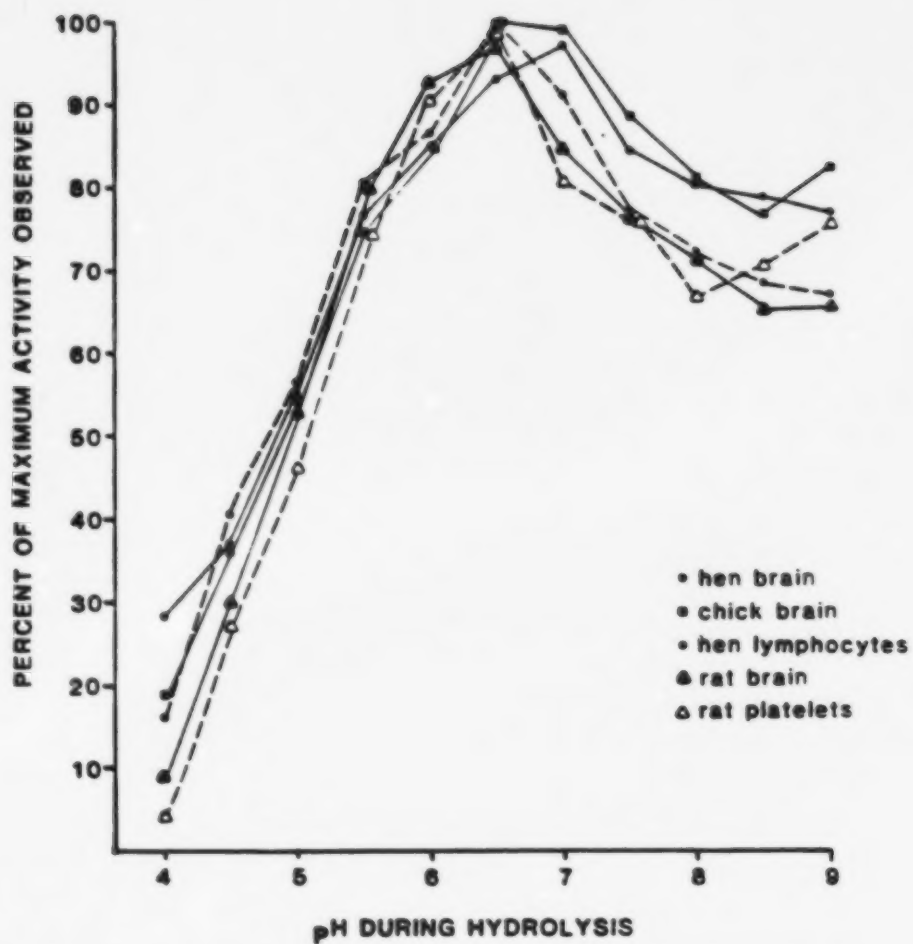


Figure 27. The effect of pH on NTE activity at high ionic strength.



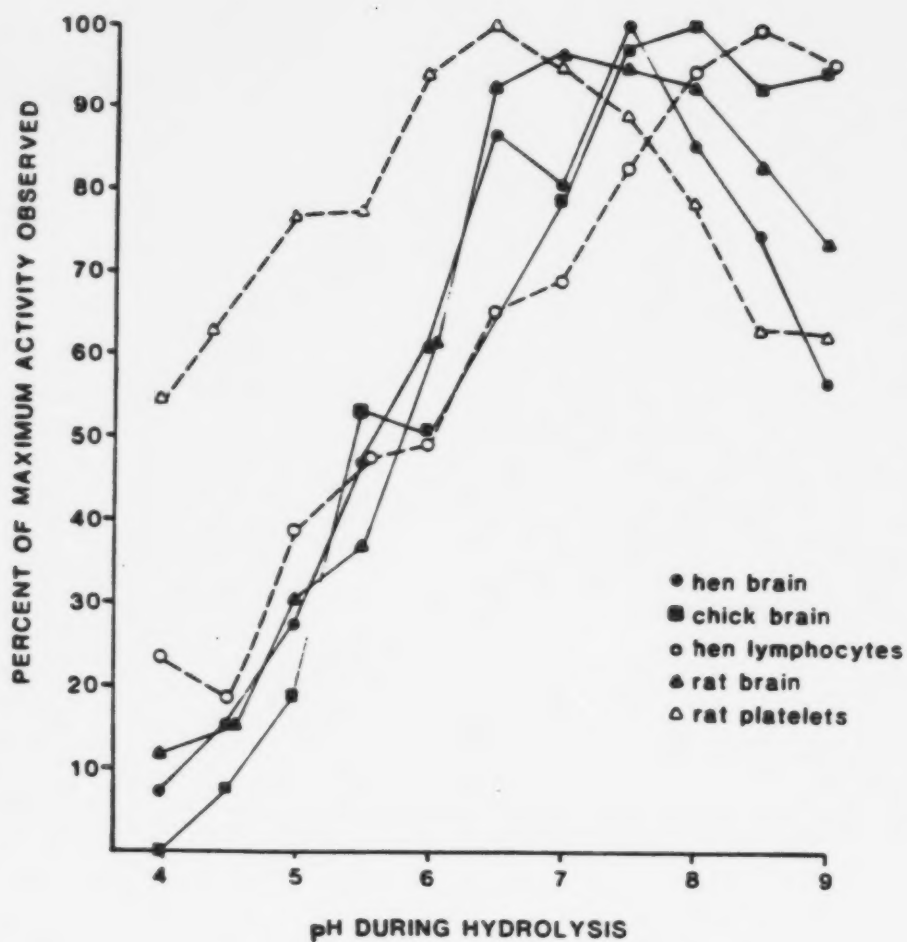


Figure 28. The effect of pH on IRE activity at high ionic strength.

## SECTION 7

### CORRELATION OF NTE ACTIVITY IN BRAIN AND LYMPHOCYTES OF THE ADULT HEN FOLLOWING DOSING WITH NEUROTOXIC AND NON-NEUROTOXIC COMPOUNDS

Since the tissue distribution study (SECTION 5) revealed the presence of NTE in peripheral lymphocytes, and since this enzyme has biochemical characteristics very similar to NTE in brain (SECTION 6), the possibility of using lymphocyte NTE activity as an indicator of CNS NTE activity was explored. Figure 29 shows the dose-response curves at 24 hr for hen brain and lymphocyte NTE activity following oral dosing with 0 to 200 mg/kg tri-ortho-cresyl phosphate (TOCP). Each point represents the mean  $\pm$  SE from three experiments. The curves appear similar; both have an ED<sub>50</sub> of approximately 40 mg/kg. The correlation coefficient for a plot of % NTE inhibition in brain versus lymphocytes is 0.92. This suggests that lymphocyte NTE might be used to monitor brain NTE activity and detect neurotoxic exposure in the hen.

This correlation was expanded to include a variety of neurotoxic and non-neurotoxic compounds. Compounds that produce delayed neuropathy without the need for metabolic activation included di-iso-propyl phosphorofluoridate (DFP), dibutyl dichlorvos (dibutyl DCV), and dipentyl dichlorvos (dipentyl DCV). Compounds requiring metabolic activation to produce neuropathy were tri-o-cresyl phosphate (TOCP), O-ethyl-O-p-nitrophenyl phenylphosphorothioate (EPN), and leptophos (O-methyl O-4-bromo-2,5-dichlorophenyl phenylphosphonothioate). The compound phenylmethanesulfonyl fluoride (PMSF) inhibits NTE but does not "age" and therefore does not produce neuropathy (Johnson, 1975; Lotti and Johnson, 1978). Compounds which are not neuropathic because they do not inhibit brain NTE activity included dichlorvos (DCV; 0,0-dimethyl 2,2-dichlorovinyl phosphate), paraoxon (PO; 0,0-diethyl O-p-nitrophenyl phosphate), Guthion<sup>TM</sup> (azinthiophosphorothioate), and malathion (0,0-dimethyl-S-[1,2-bis(ethoxycarbonyl) ethyl] phosphorodithioate). DFP, DCV, dibutyl DCV, dipentyl DCV, PO, EPN and Guthion<sup>TM</sup> are acutely toxic due to cholinesterase inhibition. Hens dosed with these compounds were also given 20 mg/kg atropine and 0.15 mg/kg physostigmine subcutaneously 10-15 min before the toxic compound and as needed after dosing. All therapeutic compounds were made up in 0.9% NaCl to give a maximum sc dose of 0.4 ml and a maximum ip dose of 1.0 ml/bird. EPN required treatment with atropine only.

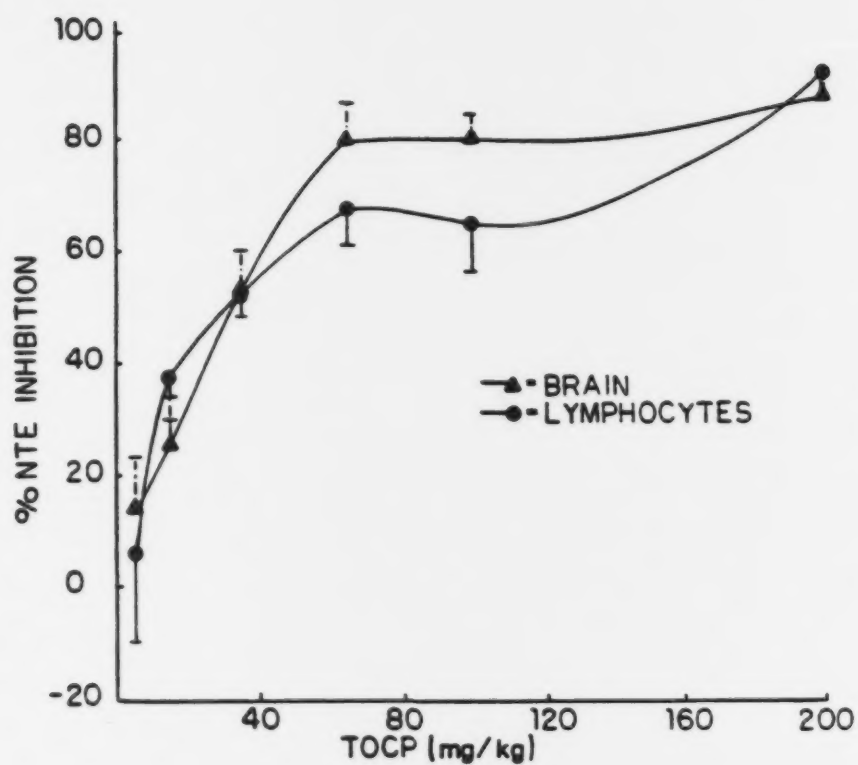


Figure 29. Dose-response curves for hen brain and lymphocyte NTE following dosing with tri-o-cresylphosphate (TOCP). Points are mean  $\pm$  SE, n = 3.

TOCP, leptophos, malathion and PMSF required no therapeutic treatment. Therapeutic compounds were given to control birds identically as to experimental birds. Treatment with therapeutic or reactivating compounds does not influence the course or severity of OP delayed neurotoxicity (Johnson, 1975a).

Hens given DFP and PO were sacrificed 24 hr after dosing. Brain and lymphocyte NTE activity for these birds is shown in Table 8. Each table entry represents a single bird. It is apparent that lymphocyte NTE does not distinguish as well as brain NTE between the neurotoxic (DFP) and non-neurotoxic (paraoxon) compound. Therefore, the brain-lymphocyte NTE correlation does not appear to hold under these conditions for at least one non-neurotoxic compound.

TABLE 8. BRAIN AND LYMPHOCYTE NTE INHIBITION  
AT 24 HOURS POST DOSE

| COMPOUND | DOSE<br>mg/kg | % NTE INHIBITION |             |
|----------|---------------|------------------|-------------|
|          |               | BRAIN            | LYMPHOCYTES |
| DFP      | 0.0           | 0.0              | 0.0         |
|          | 2.0           | 89.4             | 52.9        |
|          | 2.0           | 84.9             | 78.5        |
| Paraoxon | 0.0           | 0.0              | 0.0         |
|          | 2.0           | 7.8              | 10.7        |
|          | 5.0           | 8.5              | 44.1        |
|          | 8.0           | 3.9              | 51.8        |
|          | 10.0          | 3.9              | 19.6        |

All values are single determinations.

Since paraoxon and DFP are both potent cholinesterase inhibitors, the birds used in this study became violently ill within minutes of dosing and showed the acute effects of cholinesterase poisoning: lacrimation, fasciculation, fibrillation, and convulsions. Occasionally a dosed hen, despite therapeutic treatment with atropine, physostigmine, and 2-PAM, died. Death could reasonably be attributed to asphyxiation (DeCandole *et al.*, 1953), as the comb and wattles were dark blue, indicating anoxia. In addition, because of their debilitated condition, dosed birds which survived did not eat or drink during the following 24 hr period. Since they had been fasted for 16 hr prior to dosing, this resulted in a total fast of approximately 40 hr. The non-correlative response of lymphocyte NTE from paraoxon-dosed birds at 24 hr could be due to the effects of stress-induced, physiologically increased levels of ACTH (adrenocorti-

cotropic hormone) on lymphoid cells. The functional status and the population distribution of lymphatic cells in the hen, as in mammals, is strongly and rapidly influenced by ACTH levels via the adrenal cortical tissue. Stress, starvation, anoxia, and ACTH have been shown to have profound effects on leukocytes of hens within 24 hr (Newcomer, 1958; Wolford and Ringer, 1962). Stress, while caused by both compounds, could be an overriding factor in addition to the effects of individual inhibitory compounds on lymphoid esterases.

Since these stressors were present for 24 hr, one remedy could be to decrease the time before assay during which the noxious stimuli acted. Johnson's work had shown that 4 hr was sufficient time to label NTE with [<sup>32</sup>P] DFP (Johnson, 1969b): therefore dosing was repeated with paraoxon and DFP, and NTE was assayed 4 hr later. The results are shown in Table 9. These data suggest that at 4 hr post dose brain and lymphocyte NTE inhibition correlate well when birds are dosed with neurotoxic compounds and that lymphocyte NTE can distinguish between neurotoxic and non-neurotoxic compounds via a stimulation in activity when birds are dosed with non-neurotoxic compounds. Four hours post dose was therefore adopted as the time before assay for the remainder of the study. While a clear explanation of the inability of lymphocyte NTE to differentiate between neurotoxic and non-neurotoxic compounds at 24 hr remains to be found, the reduction from 24 to 4 hr before assay increased the practicality of measuring lymphocyte NTE in the screening of compound for delayed neurotoxicity potential -- birds dosed with acutely toxic compounds often required constant attention during the 24 hr period between dosing and assay. The non-correlative response at 24 hr, however, could caution against the use of lymphocyte NTE as a monitor of neurotoxic exposure on a chronic basis or at times much longer than 4 hr post exposure.

TABLE 9. BRAIN AND LYMPHOCYTE NTE INHIBITION  
AT 4 HOURS POST DOSE

| COMPOUND | DOSE<br>mg/kg | % NTE INHIBITION |             |
|----------|---------------|------------------|-------------|
|          |               | BRAIN            | LYMPHOCYTES |
| DFP      | 0.0           | 0.0              | 0.0         |
|          | 3.4           | 99.2             | 93.1        |
|          | 6.8           | 98.5             | 98.3        |
| Paraoxon | 5.0           | 0.0              | -59.4*      |
|          | 10.0          | 11.4             | -34.6       |

\*Negative sign indicates stimulation.



The stimulation of lymphocyte NTE activity at 4 hr with non-neurotoxic paraoxon remained unexplained. However, stimulation of cholinesterase activity by low doses of non-neurotoxic organophosphorus compounds in vivo had been observed in rat brain, liver, and kidney (Mendoza, 1976). It must be assumed that this stimulation is a physiological response, as no stimulation was observed in the in vitro exposure of blood lymphocytes to non-neurotoxic compounds during the differential titrations (Fig. 13).

The response of brain and lymphocyte NTE, at 4 hr, to the other neurotoxic compounds chosen to expand the correlation is shown in Figure 30. Dosing with each compound was repeated three times and the results are shown as mean + SE. The correlation coefficient for a plot of percent inhibition of lymphocyte NTE versus percent inhibition of brain NTE is 0.85. This result shows that lymphocyte NTE is a good monitor of brain NTE and can be used as a biomonitor of delayed neurotoxicity exposure and potential in hens under these conditions.

The compounds showing the greatest variation in response, i.e., NTE inhibition, were EPN and leptophos. In addition, since these compounds were given at neurotoxic doses (Ohkawa et al., 1977; Abou-Donia and Preissig, 1976), 70-80% NTE inhibition was expected. These variations could be due to the requirement for metabolic activation for these compounds and their optical activity: they can be discussed in terms of three factors. First, the metabolic capability of the hens used in this study could be different from those in other studies with these compounds. Second, since the stereo-isomer ratio of these compounds was not known with certainty, variations in response could be due to differential metabolism, transport, and toxicity of one of the isomers - (-)-EPN is reported to be more neurotoxic than (+)-EPM (Ohkawa et al., 1977). Third, four hours post dose may not be long enough for complete metabolism, absorption, and distribution of these compounds.

The results of dosing with non-neurotoxic compounds is shown in Figure 31. Each compound was used three times. Results are expressed as mean + SE. It is apparent that these compounds cause a stimulation of NTE activity, i.e., there is a dichotomy of response in lymphocyte NTE to neurotoxic and non-neurotoxic compounds. This point is made emphatically by the data shown in Figure 32. Here, for comparative purposes, the response of brain and lymphocyte NTE is replotted for the homologous series DCV (non-neurotoxic), dibutyl DCV (neurotoxic), and dipentyl DCV (neurotoxic). Each homologue was given at equimolar doses. This response suggests that lymphocyte NTE, if used for screening compounds, may yield a simple "yes/no" answer to the question of delayed neurotoxicity potential.

The data presented in SECTIONS 6 and 7 do not demonstrate conclusively that hen brain NTE and the enzyme operationally defined as NTE in lymphocytes are identical. Proof of this con-

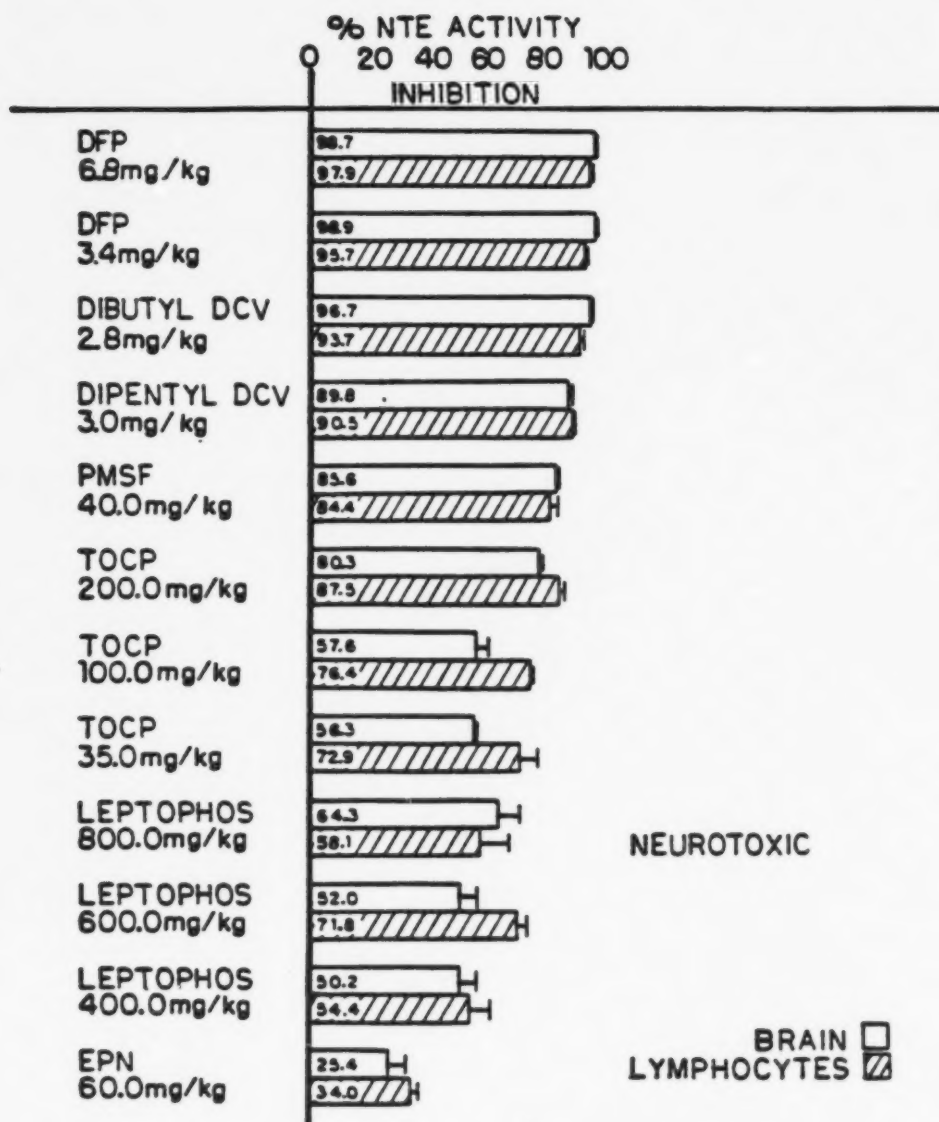


Figure 30. The inhibition of NTE activity in brain and lymphocytes from hens dosed with neurotoxic compounds. Mean  $\pm$  SE, n = 3.

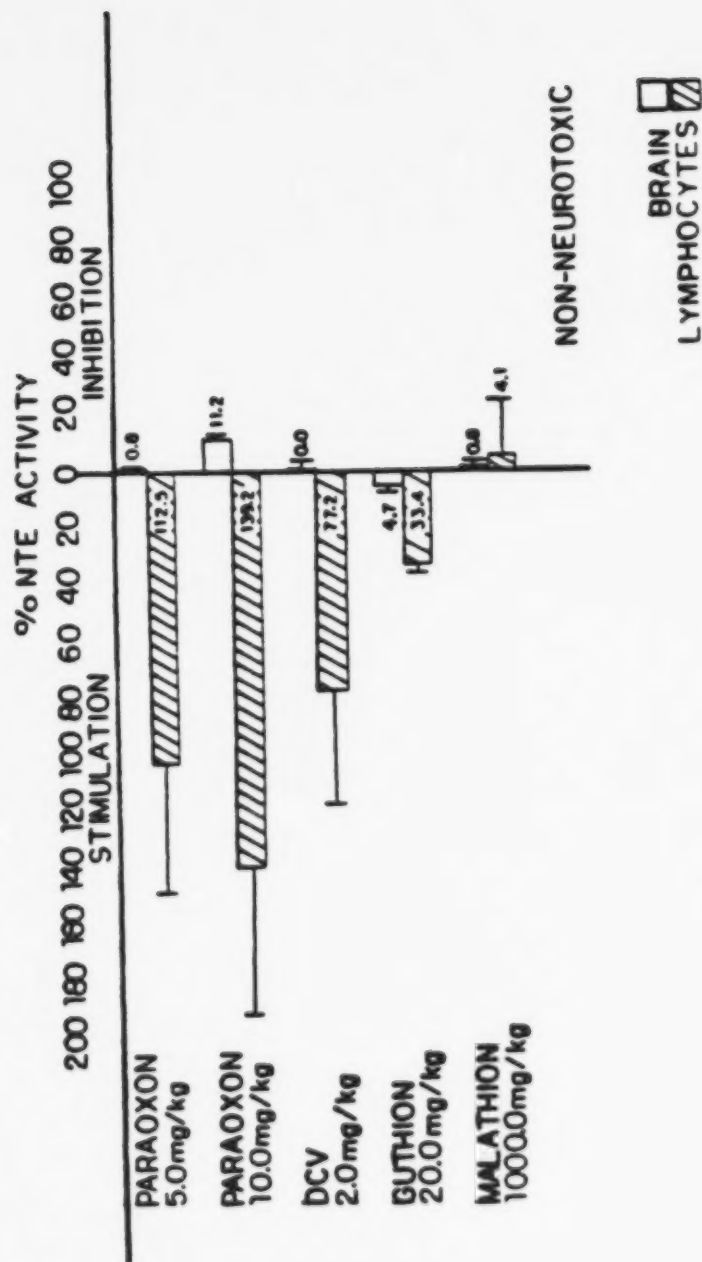


Figure 31. NTE activity in brain and lymphocytes from hens dosed with non-neurotoxic compounds. Mean  $\pm$  SE,  $n = 3$ .

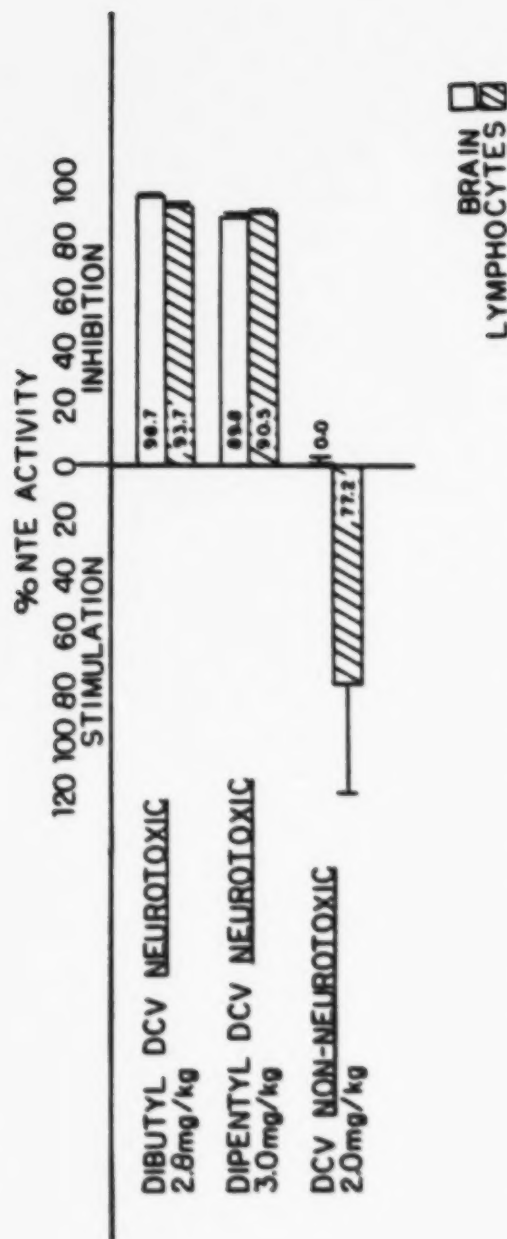


Figure 32. NTE activity in brain and lymphocytes from hens dosed with neurotoxic and non-neurotoxic compounds. Mean  $\pm$  SE,  $n = 3$ .

tention must await further work on isolation of the enzyme from both tissue sources. However, the similarities in response to heat, pH, substrates and inhibitors in vitro, as well as a similar response to neurotoxic organophosphorus compounds 4 hr after dosing support the idea that NTE exists in lymphocytes as well as in neural tissue, and can be used as an indicator of neurotoxicity.



## SECTION 8

### SUBCELLULAR FRACTIONATION

Subcellular fractionation of hen brain was carried out using the basic methodology and terminology of Whittaker and Baker (1972). The objective of this study was the identification of a fraction having higher specific activity for NTE than whole homogenate. Up to 70% of the NTE in brain was recovered in the microsomal fraction ( $P_3$ ), with an enrichment of approximately two-fold with respect to the parent homogenate. This fraction was consequently used as the source of NTE for solubilization and ultimately, immobilization in order to maximize yield. Fractionation for the purpose described above was actually part of a larger objective, the localization of NTE within the cell. This objective was part of a complementary project sponsored by NIEHS. The results of the entire study have been published (Richardson et al., 1979).

Attempts to isolate the enzyme in greater quantity from larger tissue sources (ovine and bovine brain) are discussed in SECTION 10. NTE has been identified in other tissues of the hen (SECTION 5). However, the activity per g tissue and quantity of tissue readily available make it unlikely that any of these tissues would be a useful source of NTE for purification or immobilization.

## SECTION 9

### SOLUBILIZATION

Hen brain NTE is found primarily in a microsomal fraction. In order for classical enzyme purification methods to be employed, this membrane bound activity had to be solubilized. The work described in this section was supported primarily by NIH Grant ES01611, but the results are included here since they are relevant to work described in other sections of this report.

#### METHODS

Microsomal NTE was obtained as described in SECTION 11. The microsomal pellet was then resuspended in an appropriate detergent suspension. An aliquot of this microsome-detergent suspension was saved and the remainder centrifuged at  $100,000 \times G_{avg}$  for 60 min. The resulting clear supernatant was carefully pipetted off and designated soluble NTE. All fractions - microsomes, microsomes plus detergent, supernatant (i.e., soluble), and pellet - were assayed for NTE activity. When studying the effect of lipid on NTE activity, 4 mg/ml asolectin was included in the solubilizing detergent suspension.

#### DETERMINATION OF OPTIMAL SOLUBILIZATION CONDITIONS

Solubilized NTE is defined in this study as NTE activity not sedimenting at  $100,000 \times G_{avg}$  for 60 min. Table 10 shows the results of a typical solubilization experiment. Simple hand homogenization in buffer (50 mM Tris, 2 mM EDTA, pH 8.0) or buffer plus 2 M NaCl does not solubilize NTE to any extent. Homogenization with Triton X-100 will solubilize NTE although the enzyme does lose activity in the presence of detergent. The % yield of soluble NTE can be expressed either as a corrected yield, that is, the NTE activity in the supernatant divided by the microsomal activity in the presence of detergent; or the true yield, that is, the NTE activity in the supernatant divided by the microsomal activity with no detergent present. The objective of a solubilization experiment is to maximize the amount of NTE solubilized while minimizing the amount of NTE activity lost due to detergent inactivation.

Optimal solubilization conditions found for various detergents are shown in Table 11 (Davis and Richardson, 1980b). The soluble NTE yield using octyl- $\beta$ -D-glucopyranoside could probably

TABLE 10. MICROSOMAL NTE SOLUBILIZATION EXPERIMENTS

| 1<br>ADDITION                                    | 2<br>MICROSOMAL<br>NTE ACT.<br>BEFORE<br>CENTRIF. | 3<br>MICROSOMAL<br>NTE ACT.<br>WITHOUT<br>ADDITION | 4<br>SUPER.<br>NTE<br>ACT. | 5<br>PELLET<br>NTE<br>ACT. | 6<br>CORRECTED<br>YIELD<br>(col. 4/<br>col. 2) | 7<br>TRUE<br>YIELD<br>(col. 4/<br>855) |
|--------------------------------------------------|---------------------------------------------------|----------------------------------------------------|----------------------------|----------------------------|------------------------------------------------|----------------------------------------|
| None                                             | 855                                               | ---                                                | 32.5                       | 822.5                      | 3.8                                            | 3.8                                    |
| NaCl (2 M)                                       | 836                                               | 97.8                                               | 14.8                       | 713.0                      | 1.8                                            | 1.7                                    |
| Triton X-100<br>(% Det./% Prot.<br>Ratio = 0.82) | 590                                               | 60.9                                               | 389.0                      | 201.0                      | 65.9                                           | 45.5                                   |

NTE units are nmol phenol/min/12 ml final volume. See text for experimental details and definition of true and corrected yield.

TABLE 11. OPTIMAL SOLUBILIZATION CONDITIONS AND RESULTS FOR VARIOUS DETERGENTS

| DETERGENT                                                | BUFFER                              | PROTEIN/<br>DETERGENT RATIO | % CORRECTED YIELD | % TRUE YIELD |
|----------------------------------------------------------|-------------------------------------|-----------------------------|-------------------|--------------|
| Triton X-100<br>(non-ionic)                              | 10 mM Tris<br>0.4 mM EDTA<br>pH 8.0 | 0.44 - 0.82                 | 60 - 66           | 47 - 49      |
| Sodium Cholate<br>(ionic)                                | 50 mM Tris<br>2 mM EDTA<br>pH 8.0   | 5.9 - 6.8                   | 69                | 46           |
| Octyl- $\beta$ -D-Gluco-<br>pyranoside<br>(zwitterionic) | 50 mM Tris<br>2 mM EDTA<br>pH 8.0   | 5.2                         | 32                | 20           |

55

For definitions of true and corrected yield, see text.

be improved by using a lower ionic strength buffer but as we did not plan to routinely use this system, we did not optimize it further.

#### INHIBITOR CHARACTERISTICS OF SOLUBLE NTE

Titration curves of sodium cholate or Triton X-100 solubilized NTE resembled those of the membrane-bound enzyme, indicating that NTE does not change its inhibitor characteristics during the solubilization process (Davis *et al.*, 1980).

Asolectin (4 mg/ml) prevents the loss of NTE activity when included in the solubilization procedure. This suggests that NTE is a lipid-dependent, intrinsic membrane protein (Coleman, 1973).

#### PH-ACTIVITY CURVES FOR NATIVE AND SOLUBLE NTE

pH-activity relationships were determined for particulate NTE in whole brain homogenates, microsomes, and NTE solubilized from microsomes with Triton X-100. The methodology used was that described for brain, lymphocyte, and platelet NTE in SECTION 6 except that no KCl was used in these experiments.

pH readings taken from a duplicate set of samples at the stages of PV hydrolysis and color development are given in Table 12. Whole brain homogenate was used in this experiment. Even at 100 mM, the buffers of interest did not completely override the 10 mM pH 8.0 Tris used in the inhibition step of the assay. The pH at the time of color development was between 8.0 and 9.0 for all samples. This corresponds to the optimal pH range shown in Table 5. Blank samples were made up for each pH system.

As for NTE in whole brain homogenate, the optimal pH region for microsomal and solubilized NTE is quite broad, between 6.5 and 9.0, with apparent peak activity occurring between 6.5 and 7.0 for microsomal NTE and between 7.0 and 7.5 for soluble NTE (Table 13 and Fig. 33). Both soluble and particulate NTE were more sensitive to acid pH than to alkaline pH. Since solubilization of membrane bound NTE is the initial step in purification of the enzyme, it is encouraging that the solubilization process does not appreciably alter the pH characteristics of the enzyme.

#### PH DENATURATION OF NATIVE AND SOLUBLE NTE

The effects of pH on the short term stability of particulate and soluble NTE was determined as another check on the behavior of the enzyme after solubilization. A 10% whole brain homogenate made in water instead of buffer was diluted with 50 mM buffers from pH 4.0 to 11.0, and let stand at room temperature for 80 min. At this time 0.5 ml aliquots of homogenate were added to 1.5 ml 50 mM Tris 2 mM EDTA, pH 8.0, containing inhibitors as usual for the standard NTE assay. The pH of the assay mixture at the time of phenyl valerate hydrolysis was measured in an additional set



TABLE 12. pH-ACTIVITY RELATIONSHIPS FOR HEN BRAIN  
NTE AND IRE IN WHOLE HOMOGENATE (LOW IONIC STRENGTH)

| BUFFER  | pH <sup>a</sup> | MEASURED<br>pH FOR PV<br>HYDROLYSIS <sup>a</sup> | pH DURING<br>COLOR<br>DEVELOPMENT <sup>b</sup> | ENZYME ACTIVITY,<br>% OF MAXIMUM |       |
|---------|-----------------|--------------------------------------------------|------------------------------------------------|----------------------------------|-------|
|         |                 |                                                  |                                                | NTE                              | IRE   |
| TRIS    | 9.0             | 8.71                                             | 8.79                                           | 92.9                             | 95.3  |
|         | 8.5             | 8.33                                             | 8.71                                           | 99.8                             | 96.3  |
|         | 8.0             | 7.99                                             | 8.60                                           | 100.0                            | 100.0 |
|         | 7.5             | 7.54                                             | 8.48                                           | 97.4                             | 97.9  |
|         | 7.0             | 7.04                                             | 8.42                                           | 93.4                             | 90.6  |
| MES     | 6.5             | 6.57                                             | 8.58                                           | 91.3                             | 85.3  |
|         | 6.0             | 6.08                                             | 8.43                                           | 83.0                             | 52.9  |
|         | 5.5             | 5.59                                             | 8.32                                           | 79.8                             | 40.8  |
| Acetate | 5.0             | 5.16                                             | 8.50                                           | 64.9                             | 30.4  |
|         | 4.5             | 4.65                                             | 8.41                                           | 56.2                             | 22.0  |
|         | 4.0             | 4.22                                             | 8.27                                           | 45.2                             | 18.8  |

<sup>a</sup>At 37°C, the temperature of the assay.

<sup>b</sup>Corrected to 25°C.

TABLE 13. pH-ACTIVITY RELATIONSHIPS FOR PARTICULATE AND SOLUBILIZED NTE AND IRE FROM HEN BRAIN AT LOW IONIC STRENGTH

| BUFFER  | pH* | WHOLE<br>HOMOGENATE |       | MICROSOMES |       | SOLUBILIZED |       |
|---------|-----|---------------------|-------|------------|-------|-------------|-------|
|         |     | NTE                 | IRE   | NTE        | IRE   | NTE         | IRE   |
| TRIS    | 9.0 | 92.9                | 95.3  | 96.1       | 100.0 | 85.0        | 97.9  |
|         | 8.5 | 99.8                | 96.3  | 97.0       | 88.9  | 89.9        | 82.2  |
|         | 8.0 | 100.0               | 100.0 | 95.1       | 83.7  | 85.0        | 92.1  |
|         | 7.5 | 97.4                | 97.9  | 96.3       | 78.3  | 100.0       | 93.4  |
|         | 7.0 | 93.4                | 90.6  | 98.3       | 81.5  | 91.9        | 100.0 |
| MES     | 6.5 | 91.3                | 85.3  | 100.0      | 77.3  | 89.7        | 89.4  |
|         | 6.0 | 83.0                | 52.9  | 91.8       | 56.9  | 82.5        | 66.7  |
|         | 5.5 | 79.8                | 40.8  | 89.6       | 47.3  | 72.5        | 47.7  |
| Acetate | 5.0 | 64.9                | 30.4  | 76.1       | 49.2  | 70.7        | 45.7  |
|         | 4.5 | 56.2                | 22.0  | 57.8       | 37.7  | 57.0        | 38.6  |
|         | 4.0 | 45.2                | 18.8  | 47.4       | 18.2  | 45.6        | 20.9  |

\*At 37°C.

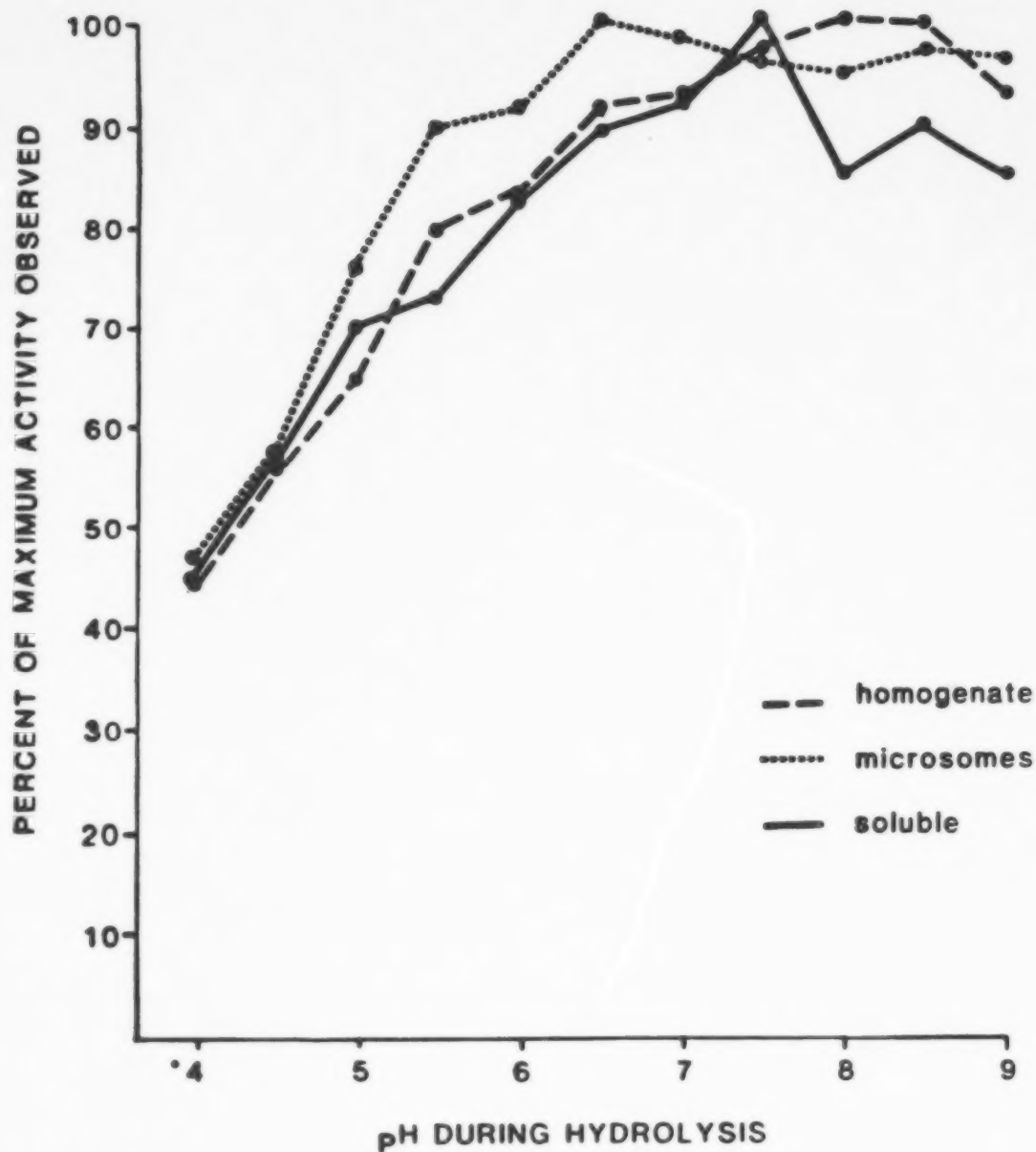


Figure 33. pH activity curves at low ionic strength for hen brain NTE in whole homogenate, resuspended microsomes, and after solubilization with Triton X-100.

of samples made up for that purpose and is given in Table 14. pH during substrate hydrolysis ranged from 6.78 to 7.84. The activity of the enzyme probably varies less than 10% in this range (Table 12). The much larger changes in activity seen in this experiment, therefore, are attributable to the pH of the incubation mixture before the assay. The storage pH given in the table was not verified by direct measurement in this experiment, but was measured in similar experiments done with microsomal and solubilized NTE (Tables 15 and 16). The maximum deviation from nominal pH was 0.15 units, seen at the extremes of the range. The SDS-AAP for the experiment using homogenate was made up in the standard 50 mM Tris - 2 mM EDTA, pH 8.0. pH at the time of color development was also determined, and it may not have been in the optimal range of 8.0 - 9.0 for all samples. The deviation from maximum color development could be significant (10%) in the last 5 or 6 samples. However, the overall shape of the storage pH-activity curve should be valid (Table 14). A plot of % maximal activity vs. pH of storage shows an interesting bimodal distribution with peaks at 9.0 and 4.0 (Fig. 34). The activity profile for IRE was much broader, and much less inactivation at low pH was seen (Fig. 35).

A similar experiment was done with microsomal NTE. A 10% hen brain homogenate made in water (i.e., unbuffered) was centrifuged at 9,500 x g<sub>avg</sub> for 20 min. The supernatant was removed and kept cold while the pellet was homogenized again in water, and centrifuged as before. Microsomes were pelleted from the combined supernatants at 78,000 x g<sub>avg</sub> for 90 min. The pellets were resuspended in 6 ml H<sub>2</sub>O per g whole brain equivalent, and then diluted 1:19 with H<sub>2</sub>O to give a working suspension for assay. This suspension was diluted 3:2 with buffers over the range of 11.0 to 3.0 as described earlier. The microsomal resuspensions at pH 3.5, 4.0, 4.5, and 5.0 formed precipitates which redissolved when the pH was raised by the addition of 0.2 M Tris-EDTA, pH 8.0, at the beginning of the assay. The assay was carried out as before, except that storage time was decreased to 60 min and the pH of the enzyme-buffer resuspension before and after the addition of 0.2 M Tris - 4 mM EDTA, pH 8.0, was measured in an extra set of samples made up for that purpose. In addition, the SDS-AAP used to stop the reaction was made up in 0.2 M Tris, pH 8.5. This insured that the pH of the assay solution would be in the optimal range of 8.0 - 9.0 for color development (see data in Tables 15 and 16). The data from this experiment are shown in Table 15 and Figures 34 and 35. Microsomal NTE was much more resistant to low pH than NTE in whole homogenate. NTE lability in whole homogenate might be the result of degradation by lysosomal acid proteases which are largely absent in a microsomal preparation. Microsomal IRE showed a moderate increase in stability between pH 6.0 and 9.0 compared to IRE in whole homogenate, but its behavior at low pH was unchanged.

TABLE 14. THE EFFECT OF pH ON THE SHORT TERM STABILITY  
OF NATIVE (WHOLE BRAIN HOMOGENATE) NTE AND IRE

| BUFFER              | STORAGE<br>pH* | pH DURING<br>SUBSTRATE<br>HYDROLYSIS* | % MAXIMUM ACTIVITY |       |
|---------------------|----------------|---------------------------------------|--------------------|-------|
|                     |                |                                       | NTE                | IRE   |
| CAPS                | 11.0           | 7.84                                  | 0.4                | 21.7  |
|                     | 10.5           | 7.72                                  | 7.3                | 40.3  |
|                     | 10.0           | 7.57                                  | 82.3               | 83.0  |
| BIS-TRIS<br>PROPANE | 9.5            | 7.76                                  | 80.3               | 78.3  |
| TRIS                | 9.0            | 7.68                                  | 100.0              | 100.0 |
|                     | 8.0            | 7.49                                  | 27.8               | 78.3  |
|                     | 7.0            | 7.32                                  | 31.4               | 90.7  |
| MES                 | 6.0            | 7.14                                  | 7.1                | 86.8  |
| Acetate             | 5.0            | 7.21                                  | 12.9               | 62.0  |
|                     | 4.5            | 7.06                                  | 15.7               | 55.0  |
|                     | 4.0            | 6.78                                  | 17.7               | 51.0  |

\*Corrected to 25°C.



TABLE 15. THE EFFECT OF pH ON SHORT TERM STABILITY  
OF MICROSOMAL NTE AND IRE

| BUFFER  | pH*  | MICROSOMES<br>+ BUFFER, pH* | pH OF<br>ASSAY* | % MAXIMUM ACTIVITY |       |
|---------|------|-----------------------------|-----------------|--------------------|-------|
|         |      |                             |                 | NTE                | IRE   |
| CAPS    | 11.0 | 10.85                       | 8.16            | 4.2                | 23.6  |
|         | 10.5 | 10.42                       | 8.11            | 58.3               | 70.9  |
|         | 10.0 | 9.95                        | 8.07            | 100.0              | 87.4  |
| TRIS    | 9.0  | 8.92                        | 8.04            | 96.0               | 97.6  |
|         | 8.0  | 7.94                        | 7.96            | 91.8               | 93.7  |
|         | 7.0  | 6.95                        | 7.90            | 88.5               | 100.0 |
| MES     | 6.0  | 6.00                        | 7.93            | 89.9               | 89.8  |
| Acetate | 5.0  | 5.04                        | 7.99            | 49.1               | 60.6  |
|         | 4.5  | 4.53                        | 7.94            | 65.6               | 53.5  |
|         | 4.0  | 4.07                        | 7.89            | 75.2               | 40.9  |
| Citrate | 3.5  | 3.59                        | 7.60            | 7.5                | 19.7  |
|         | 3.0  | 3.12                        | 7.48            | 1.2                | 12.6  |

\*Corrected to 25°C.

TABLE 16. THE EFFECT OF PH ON SHORT TERM STABILITY OF SOLUBLE NTE AND IRE

| BUFFER  | PH*  | ACTUAL PH* DURING |                         |                      | % MAXIMAL ACTIVITY |          |       |
|---------|------|-------------------|-------------------------|----------------------|--------------------|----------|-------|
|         |      | STORAGE           | SUBSTRATE<br>HYDROLYSIS | COLOR<br>DEVELOPMENT | NTE                |          | IRE   |
|         |      |                   |                         |                      | 1st EXP.           | 2nd EXP. |       |
| CAPS    | 11.0 | 10.88             | 8.30                    | 8.39                 | 1.8                | 3.1      | 25.5  |
|         | 10.5 | 10.45             | 8.19                    | 8.37                 | 36.9               | 53.7     | 66.8  |
|         | 10.0 | 9.97              | 8.08                    | 8.34                 | 72.6               | 72.8     | 81.3  |
| B-T-P   | 9.5  | 9.50              | 8.23                    | 8.40                 | 80.9               | ----     | 91.3  |
|         | 9.0  | 8.98              | 8.14                    | 8.35                 | 81.1               | 84.6     | 89.4  |
| TRIS    | 8.0  | 7.99              | 7.97                    | 8.34                 | 85.0               | 84.8     | 91.9  |
|         | 7.0  | 7.01              | 7.84                    | 8.29                 | 89.5               | 88.2     | 100.0 |
|         | 6.0  | 6.01              | 7.71                    | 8.30                 | 93.5               | 99.1     | 86.8  |
| Acetate | 5.0  | 5.04              | 7.81                    | 8.33                 | 99.8               | 94.6     | 68.1  |
|         | 4.5  | 4.54              | 7.64                    | 8.31                 | 100.0              | 100.0    | 67.1  |
|         | 4.0  | 4.08              | 7.52                    | 8.32                 | 98.8               | 99.8     | 61.9  |
| Citrate | 3.5  | ----              | ----                    | ----                 | ----               | 5.0      | ----  |
|         | 3.0  | ----              | ----                    | ----                 | ----               | 0.0      | ----  |

\*At 25°C.

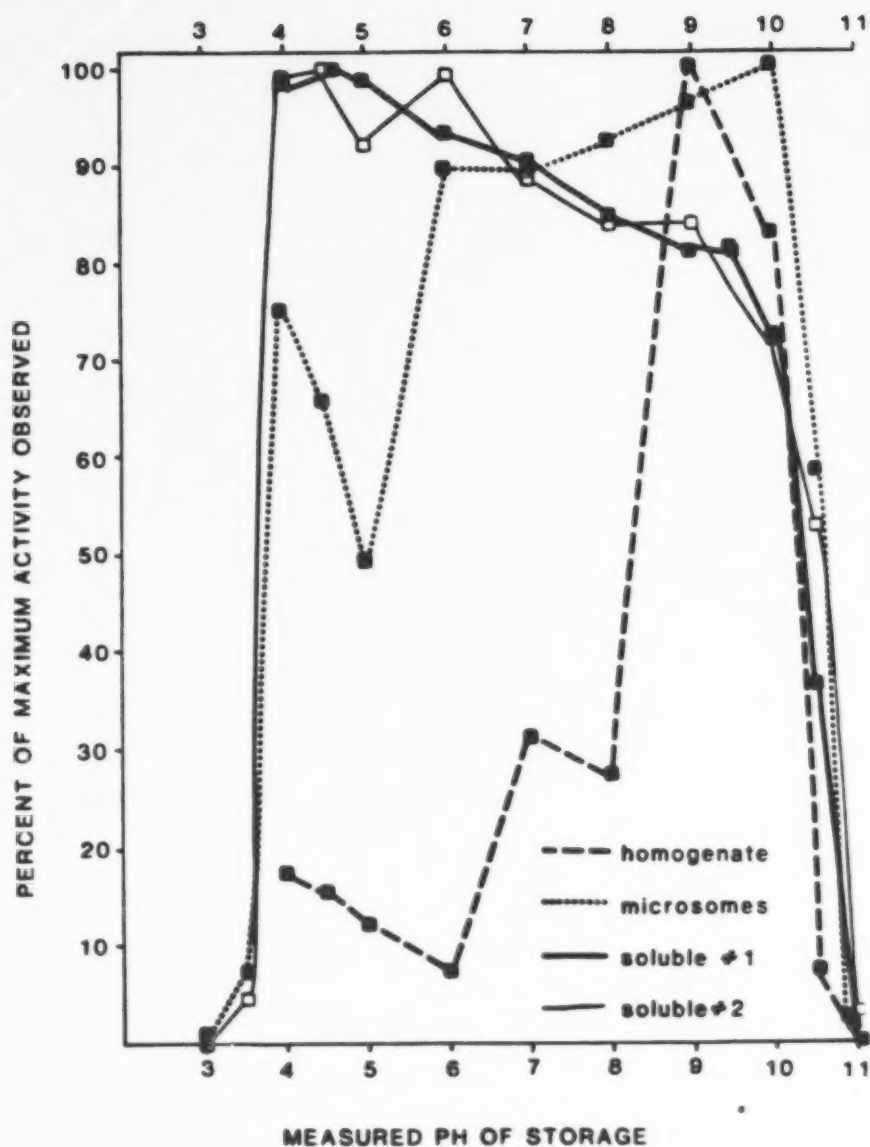


Figure 34. The effect of pH on the stability of hen brain NTE in whole homogenate, resuspended microsomes, and after solubilization with Triton X-100.

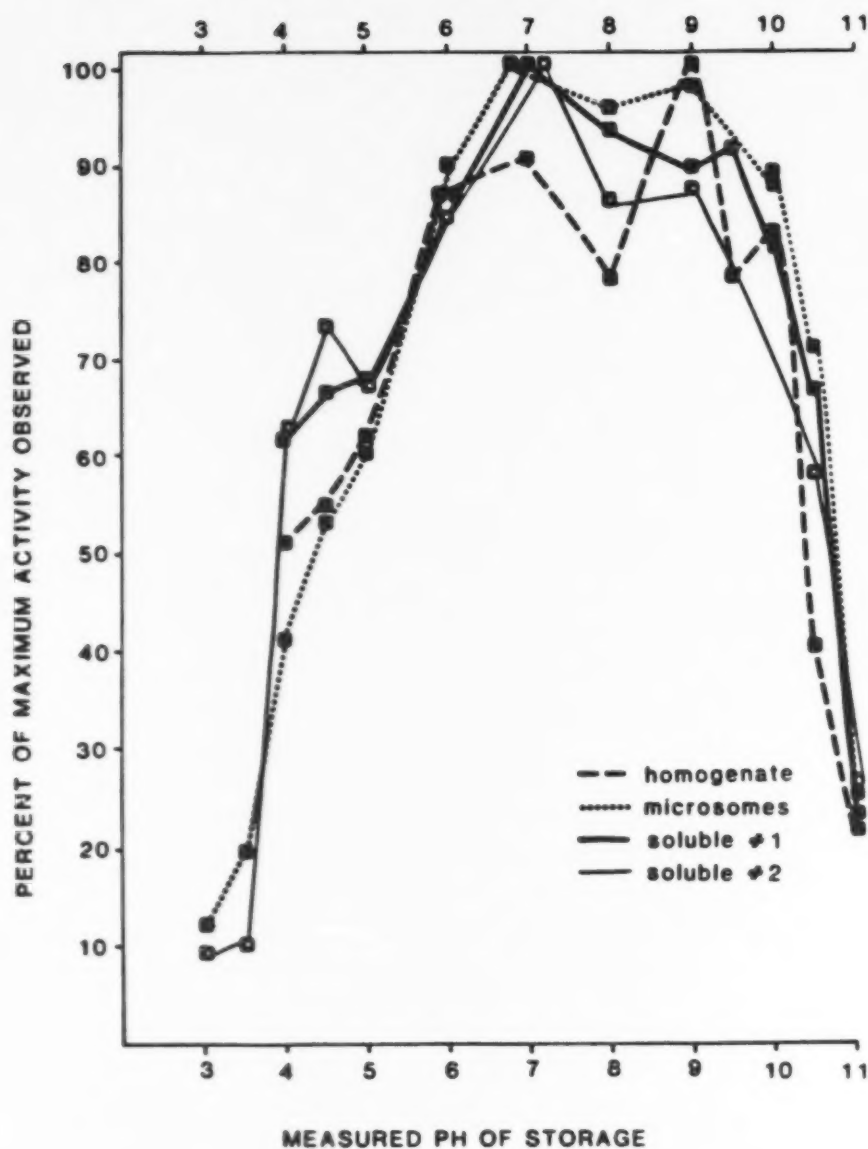


Figure 35. The effect of pH on the stability of hen brain IRE in whole homogenate, resuspended microsomes, and after solubilization with Triton X-100.

A similar experiment was done twice with soluble NTE as a check on reproducibility. Soluble NTE was made by resuspending microsomal pellets in 0.1% Triton X-100, unbuffered, and centrifuging at 78,000 x g<sub>avg</sub> for 90 min to sediment unsolubilized material. The supernatant was diluted with the buffers of interest (pH 3.0 to 11.0) to give a working enzyme preparation. These experiments were carried out essentially as described for microsomal NTE (pH adjustment for inhibition and substrate hydrolysis with 0.1 M Tris - 2 mM EDTA, pH 8.0, and for color development with SDS-AAP in 0.2 M Tris, pH 8.5). pH determinations were made on additional "assay" mixtures at the stage of storage, substrate hydrolysis, and color development for the first of these experiments. Data is given in Table 16 and in Figures 34 and 35. The agreement between duplicate experiments is quite good. Remarkably, the greatest activity was seen in NTE exposed to pH 4.0 and 4.5. Activity decreased gradually with increasing pH to pH 10.0 and then dropped rapidly. IRE was less stable than NTE below pH 6.0 but more stable at higher pH's. These results indicate that partially purified NTE is quite tolerant to pH changes. This information is useful in designing experiments involving immobilization and purification by indicating that NTE will withstand a wide range of experimental conditions.



## SECTION 10

### ATTEMPTED BULK PREPARATION OF SOLUBLE NTE

Ovine and bovine brain were investigated as starting material for the preparation of large quantities of soluble NTE. Brain homogenates from these species had approximately one-half the specific activity of hen brain. Mipaflox I<sub>50</sub>'s were 12 and 6  $\mu$ M respectively, compared to approximately 8  $\mu$ M for the hen. Activity was solubilized from both sources with unbuffered Triton X-100 according to the method used for hen brain (SECTION 9). The ovine preparation was found to be very unstable, with a half-life of 4-8 days at 4°C. The solubilized bovine preparation had a half-life of about 12 days at 4°C. When the bovine preparation was characterized by titration with mipaflox, it was found that the I<sub>50</sub> of the solubilized preparation was 60-80  $\mu$ M. Therefore, the solubilized preparation from bovine brain microsomes was either an altered NTE or an activity different from NTE.

An attempt was made to expedite the isolation of bovine brain microsomes by isoelectric precipitation (pH adjusted to 4.7) with citrate. It was found that 60-80% of the activity could be recovered that way, but the activity could no longer be solubilized with detergent.

Because of the relatively low yield, poor stability, and altered inhibitor characteristics for solubilized bovine NTE, ovine and bovine brain offer no significant advantage over hen brain as a starting material for solubilizing the enzyme. It is possible that the yield from ovine brain might be improved by using a detergent other than Triton. Stability might also be improved. To date, other solubilizing agents for ovine brain have not been investigated.

## SECTION 11

### STABILITY STUDIES WITH RESPECT TO TIME

At the beginning of this study virtually nothing was known about the stability of NTE over time. Purification of the enzyme as well as the development of an in vitro method for prediction of OP-induced delayed neuropathy would be facilitated by knowledge of the stability of the enzyme under a variety of conditions. In this section information is presented on the stability of native (particulate) and solubilized NTE prepared in several ways and stored at several temperatures.

#### METHODS

Brains were obtained from untreated white Leghorn hens or chicks sacrificed by decapitation. Whole brain homogenates (10%, w/v) were made in the following media: 50 mM Tris HCl - 2 mM EDTA, pH 8.0; 0.32 M sucrose - 10 mM Tris HCl - .4 mM EDTA, pH 8.0; unbuffered 0.32 M sucrose; and H<sub>2</sub>O.

Microsomal NTE was obtained by centrifuging whole brain homogenate at 9,500 x g<sub>avg</sub> for 20 min. The supernatant (S<sub>2</sub>) was removed, and kept cold while the pellets (P<sub>1</sub> + P<sub>2</sub>) were resuspended in the original homogenizing medium and centrifuged as before. The pooled supernatant from both spins were sedimented at 78,000 x g<sub>avg</sub> for 90 min or 116,000 x g<sub>avg</sub> for 60 min. For stability determinations, the microsomes from 1 g brain were resuspended in 10 volumes of 50 mM Tris-HCl 2 mM EDTA, pH 8.0, or H<sub>2</sub>O. NTE was solubilized by resuspending microsomes obtained from 1 g brain in 10 ml 0.1% Triton X-100 in H<sub>2</sub>O, or 1.0% sodium cholate in 50 mM Tris, 2 mM EDTA, pH 8.0 and sedimenting unsolubilized material with conditions used to obtain microsomal pellets. When studying the effect of lipid on stability, 4 mg/ml asolectin was included in the sodium cholate solubilization procedure.

For determination of enzyme stability, each homogenate, microsomal resuspension, or solubilized NTE preparation was stored as measured aliquots so that the frozen samples would be frozen and thawed only once. All frozen samples were frozen and immediately thawed for day 0 NTE determination. Samples made in each of these media were stored at 4°, -10°, -18°, or -36° until assayed. In two experiments, soluble NTE was sterilized by passage through a 0.2 µm membrane filter before storage at 4°C. Sodium cholate was removed from soluble preparations by passing it through a Sephadex<sup>TM</sup> G-25 column, and eluting with 50 mM Tris,

2 mM EDTA, pH 8.0. NTE was eluted in the void volume.

NTE activity was determined according to the standard method described in SECTION 3.

#### IMPROVING THE PRECISION OF THE ASSAY OF FROZEN HOMOGENATES

Samples of whole brain homogenate which had been frozen were found to be coarsely particulate, and tended to settle out during assay. Agreement between duplicate tubes was much poorer than that of samples which had not been frozen. The effects of rehomogenization of a frozen 10% brain homogenate on NTE activity and the precision of duplicate determinations is shown in Table 17. The absorbances recorded in the first column are from samples inhibited with 40  $\mu$ M paraoxon, in which only NTE and certain inhibitor resistant esterases (IRE) are active. The absorbances in the second column are derived from similar samples inhibited with paraoxon and also with mipafox (50  $\mu$ M) which inactivates NTE but not IRE. The mean NTE activities given in Table 17 are not significantly different at the 5% confidence level as determined by an analysis of variance testing for interaction. Rehomogenization reduced the standard error of duplicate determinations by a factor of 5 to 10, to a level of agreement identical to that seen with freshly prepared samples.

#### THE STABILITY OF WHOLE BRAIN HOMOGENATE

The percentage of NTE activity remaining in aliquots of whole brain homogenate after one day to twelve weeks storage at three temperatures is given in Table 18. Samples made in sucrose or buffered sucrose and stored at  $-36^{\circ}\text{C}$  gave the best retention of activity. These samples showed no loss of activity over the first two weeks of storage. In general, sucrose enhanced stability in frozen samples but not in samples stored at  $4^{\circ}\text{C}$ . Buffering (or the presence of ions) promoted stability in the latter case.

Samples frozen in unbuffered sucrose appeared to have more activity than a duplicate sample which was not frozen. In a second experiment designed to verify this result, the activity of frozen samples was not significantly different from aliquots of the same sample which had been frozen.

#### THE STABILITY OF MICROSOMAL NTE

Microsomal NTE had the greatest stability if it was stored as a frozen resuspension of microsomes in  $\text{H}_2\text{O}$  (Table 19). Microsomal NTE frozen in buffer was much less stable. This effect of buffering (or perhaps the presence of ions) was reversed in samples stored at  $4^{\circ}\text{C}$ . In general, microsomal NTE was somewhat more stable than NTE in whole homogenate.

TABLE 17. THE EFFECT OF REHOMOGENIZATION ON NTE ACTIVITY AND PRECISION OF THE ASSAY

| TREATMENT                   | NTE + IRE      |                   | IRE            |                   | NTE<br>ACTIVITY <sup>c</sup> |
|-----------------------------|----------------|-------------------|----------------|-------------------|------------------------------|
|                             | A <sup>a</sup> | S.D. <sup>b</sup> | A <sup>a</sup> | S.D. <sup>b</sup> |                              |
| Not<br>rehomogenized        | .398           |                   | .245           |                   |                              |
|                             | .418           |                   | .275           |                   |                              |
|                             | .412           |                   | .251           |                   |                              |
|                             | .398           | .010              | .284           | .019              | 888                          |
| Rehomogenized<br>by hand    | .398           |                   | .246           |                   |                              |
|                             | .398           |                   | .250           |                   |                              |
|                             | .400           |                   | .254           |                   |                              |
|                             | .397           | .001              | .246           | .004              | 938                          |
| Rehomogenized<br>by machine | .399           |                   | .246           |                   |                              |
|                             | .397           |                   | .245           |                   |                              |
|                             | .397           |                   | .246           |                   |                              |
|                             | .398           | .001              | .250           | .002              | 951                          |

<sup>a</sup> Absorbance at 510 nm.

<sup>b</sup> Standard deviation.

<sup>c</sup> nmol/min/g tissue.

TABLE 18. THE EFFECT OF HOMOGENIZING MEDIUM AND STORAGE TEMPERATURE ON THE STABILITY OF NTE IN 10% WHOLE BRAIN HOMOGENATE

| Day | + 4°C               |                               |                      | - 10°C |                  |         | - 36°C |                  |         |
|-----|---------------------|-------------------------------|----------------------|--------|------------------|---------|--------|------------------|---------|
|     | Buffer <sup>a</sup> | Buffered Sucrose <sup>b</sup> | Sucrose <sup>c</sup> | Buffer | Buffered Sucrose | Sucrose | Buffer | Buffered Sucrose | Sucrose |
| 0   | 100 <sup>d</sup>    | 100                           | 100                  | --     | --               | --      | --     | --               | --      |
| 1   | 101                 | 100                           | 58                   | 53     | 101              | 116     | 99     | 101              | 117     |
| 3   | 83                  | 101                           | 28                   | 33     | 96               | 103     | 96     | 102              | 114     |
| 7   | 60                  | 67                            | 13                   | 31     | 86               | 87      | 84     | 96               | 112     |
| 14  | 39                  | 27                            | 10                   | 21     | 82               | 84      | 69     | 102              | 112     |
| 28  | 26                  | --                            | --                   | 17     | 48               | 52      | 62     | 67               | 69      |
| 56  | 12                  | --                            | --                   | 17     | 52               | 61      | 52     | 80               | 78      |
| 84  | 10                  | --                            | --                   | 24     | 47               | 54      | 43     | 71               | 75      |

<sup>a</sup> 50 mM Tris - 2 mM EDTA, pH 8.0.

<sup>b</sup> 0.32 M sucrose - 10 mM Tris - 0.4 mM EDTA, pH 8.0.

<sup>c</sup> 0.32 M sucrose, unbuffered.

<sup>d</sup> Activity remaining compared to day 0 for each homogenizing medium.



TABLE 19. THE EFFECTS OF BUFFERING AND STORAGE TEMPERATURE ON THE STABILITY OF MICROSOMAL NTE

| STORAGE TIME<br>(weeks) | UNBUFFERED      |       | BUFFERED |       |
|-------------------------|-----------------|-------|----------|-------|
|                         | 4°C             | -18°C | 4°C      | -18°C |
| 1                       | 57 <sup>a</sup> | 94    | 115      | 42    |
| 2                       | 42              | 82    | 97       | 20    |
| 4                       | 2               | 89    | 80       | 14    |
| 8                       | 1               | 74    | 26       | 0     |
| 12                      | 2               | 81    | 17       | 7     |

<sup>a</sup>Table entries are % activity compared to the activity found on day 0 of the study.

## THE STABILITY OF TRITON SOLUBILIZED NTE

Data on the stability of Triton solubilized NTE are shown in Table 20. Since some aliquots of whole homogenate kept at 4°C had become contaminated with microorganisms (indicated by dashed lines at 4 to 12 weeks in Table 18), the effect of sterilization of soluble NTE by filtration was also investigated. The experiment was repeated with filtered soluble NTE kept at 4°C and soluble NTE stored frozen; results from both experiments are shown (Table 20). Sterilization increased the half-life of soluble NTE kept at 4°C from approximately 3 to 12 weeks. When stored frozen at -18°C, this preparation retained 90% or more of its activity for 12 weeks. This represents the most stable preparation of NTE obtained during the course of this study.

## THE EFFECT OF LYOPHILIZATION ON STABILITY

Preliminary experiments showed that a 0.1% Triton/H<sub>2</sub>O solution of NTE could be lyophilized with no loss of activity. A 12-week stability study was done with this material concurrently with the first experiment using soluble NTE reported in Table 20. Aliquots from a single large batch of Triton solubilized enzyme were used for both of these experiments. Data from Table 20 for aliquots stored at 4° and -18°C is repeated in Table 21 to facilitate comparison with data from the lyophilized samples. Table entries reported in this section are the average of 3 replicates, and have a standard deviation of 4% or less. However, in the case of lyophilized samples, the standard deviation for groups of 3 replicates began to increase at one week of storage. Generally thereafter, one of the three replicates would show a much lower activity than the other two. This effect was seen at all three storage temperatures. The pattern of lack of agreement between replicates suggests that something other than statistical variation is involved. That is, there may be a potentially identifiable reason why some samples were less stable than others in the same treatment group. These aberrant values were omitted from calculation of average activity given for lyophilized samples in Table 21. This is, in effect, taking the most optimistic view possible of the stability of the lyophilized samples. Even so, that stability is only marginally better than that of NTE in Triton/H<sub>2</sub>O. It appears that lyophilization offers no real advantage with respect to storage. Consequently, it did not seem worthwhile to investigate the variation in stability of lyophilized samples.

## THE STABILITY OF SODIUM CHOLATE SOLUBILIZED NTE

Microsomal NTE solubilized by sodium cholate, an ionic detergent, was much less stable than that solubilized by Triton X-100, a non-ionic detergent (Table 22). Stability was better at 4°C than at -10°C. Stability could be improved, at least for one day, by including asolectin in the solubilization procedure and could be improved even more by removing the sodium cholate

TABLE 20. THE EFFECTS OF STERILIZATION BY FILTRATION AND STORAGE TEMPERATURE  
ON THE STABILITY OF TRITON SOLUBILIZED NTE

| STORAGE TIME<br>DAYS | WEEKS | 4°C              |          | 4°C         |          | -18°C       |             |
|----------------------|-------|------------------|----------|-------------|----------|-------------|-------------|
|                      |       | SOLUBLE NTE      | FILTERED | SOLUBLE NTE | FILTERED | SOLUBLE NTE | SOLUBLE NTE |
| 0                    |       | 100 <sup>a</sup> | 89       | 100         | 86       | 100         | 100         |
| 1                    |       | 108              | 110      | ---         | 96       | ---         | ---         |
| 3                    |       | 107              | 105      | ---         | 97       | ---         | ---         |
| 7                    | 1     | 104              | 97       | 98          | 100      | 100         | 100         |
|                      | 2     | 75               | 87       | 82          | 102      | 89          | 89          |
|                      | 4     | 16               | 71       | 94          | 102      | 96          | 96          |
|                      | 8     | 3                | 63       | 68          | 97       | 88          | 88          |
|                      | 12    | 1                | 51       | 58          | 98       | 90          | 90          |

<sup>a</sup> Activity remaining compared to day 0 for each preparation.

TABLE 21. THE STABILITY OF LYOPHILIZED NTE  
AT VARIOUS STORAGE TEMPERATURES

| STORAGE TIME |       | FILTERED<br>SOLUBLE<br>NTE, 4°C | SOLUBLE<br>NTE, -18°C | LYOPHILIZED SOLUBLE NTE |     |       |
|--------------|-------|---------------------------------|-----------------------|-------------------------|-----|-------|
| DAYS         | WEEKS |                                 |                       | ROOM<br>TEMP.           | 4°C | -18°C |
| 0            |       | 89                              | 86                    | -                       | 105 | -     |
| 1            |       | 110                             | 96                    | 114                     | 106 | 108   |
| 3            |       | 105                             | 97                    | 95                      | 104 | 119   |
| 7            | 1     | 97                              | 100                   | 81                      | 118 | 122   |
|              | 2     | 87                              | 102                   | 71                      | 98  | 118   |
|              | 4     | 71                              | 102                   | 51                      | 92  | 105   |
|              | 8     | 63                              | 97                    | 35                      | 84  | 106   |
|              | 12    | 51                              | 98                    | 32                      | 62  | 106   |

Table entries are % activity compared to the freshly prepared soluble NTE (unfiltered) from which all samples were prepared on day 0.

TABLE 22. THE EFFECT OF SODIUM CHOLATE ON THE STABILITY  
OF PARTICULATE AND SOLUBLE NTE

| STORAGE TIME<br>DAYS | MICROSOMES |       | MICROSOMES +<br>SOD. CHOLATE |       | SOLUBLE<br>(SOD. CHOLATE) |       |
|----------------------|------------|-------|------------------------------|-------|---------------------------|-------|
|                      | 4°C        | -10°C | 4°C                          | -10°C | 4°C                       | -10°C |
| 1                    | 110        | 97    | 50                           | 17    | 48                        | 26    |
| 3                    | 109        | 62    | 35                           | 23    | 37                        | 31    |
| 7                    | 111        | 52    | 24                           | 8     | 19                        | 22    |
| 14                   | 106        | 17    | 15                           | 7     | 17                        | 15    |
| 30                   | 96         | 2     | 10                           | 6     | 13                        | 8     |

Numbers represent % activity remaining compared to day 0 for each preparation. All microsomal and detergent preparations are buffered with 50 mM Tris, 2 mM EDTA, pH 8.0.



detergent (Table 23). Again, stability was better at 4°C than -10°C but long-term stability is poor at either temperature. Sodium cholate is a commonly used detergent for purification of membrane-bound enzymes since it can be easily removed by dialysis or molecular sieve chromatography. For purification of NTE, however, sodium cholate does not appear to be a good choice.

TABLE 23. THE EFFECT OF ADDED LIPID AND DETERGENT REMOVAL ON THE STABILITY OF SODIUM CHOLATE SOLUBILIZED NTE

| STORAGE TIME<br>DAYS | SOLUBLE |       | SOLUBLE +<br>ASOLECTIN |       | SOLUBLE + ASOLECTIN,<br>DETERGENT REMOVED |       |
|----------------------|---------|-------|------------------------|-------|-------------------------------------------|-------|
|                      | 4°C     | -10°C | 4°C                    | -10°C | 4°C                                       | -10°C |
| 1                    | 48      | 26    | 76                     | 32    | 96                                        | 94    |
| 3                    | 37      | 31    | 55                     | 26    | 86                                        | 51    |
| 7                    | 19      | 22    | 35                     | 18    | 68                                        | 33    |
| 14                   | 17      | 15    | 12                     | 12    | --                                        | --    |
| 30                   | 13      | 8     | 4                      | 6     | 34                                        | --    |

Numbers represent % activity remaining compared to day 0 for each preparation. All preparations are buffered with 50 mM Tris, 2 mM EDTA, pH 8.0.

## SECTION 12

### IMMOBILIZATION

There are a great many methods available for the immobilization of enzymes (Zaborsky, 1973). Ionic bonding to ion exchange matrices, covalent bonding to substituent groups on a variety of supports, entrapment within gel matrices, and incorporation into synthetic polymer chains are some of the most widely used approaches. A variety of methods in the first two categories were used in the attempt to obtain an active, stable preparation of immobilized NTE. Binding to ion exchangers is one of the simplest and most gentle methods available. Reactive chemicals necessary for covalent bonding are avoided, and binding takes place at a low temperature and moderate pH. However, ionically bound enzymes are susceptible to displacement by changes in pH which neutralize the ionic groups involved in bond formation on either the matrix or the enzyme. Displacement may also occur at high salt concentrations. Covalent bonding is considered to provide a more stable attachment of the enzyme to the support, but usually a good deal of the enzyme is inactivated by the reactive chemicals used to form the covalent bond. Despite the considerable body of experience which exists for enzyme immobilization, selection of the carrier providing maximum binding capacity, adequate bond stability, and minimal enzyme inactivation is still empirical.

The first attempts at the immobilization of NTE constituted a systematic trial of the major types of commercially available ion exchangers. The following supports were used: Amberlite<sup>TM</sup> IR-120 (strongly acidic, polystyrene-sulfonic acid), Amberlite<sup>TM</sup> IRE-50 (weakly acidic, polymethacrylic acid), Amberlite<sup>TM</sup> IR-45 (weakly basic, tertiary amine substituted polystyrene), Dowex<sup>TM</sup> 1 and 2 (strongly basic, quaternary ammonium substituted polystyrene), carboxymethylcellulose (weakly acidic), diethylaminoethyl (DEAE)-cellulose, microcrystalline DEAE-cellulose (Whatman), DEAE-Selectacel<sup>TM</sup> (DEAE-cellulose sold by Brown Company), DEAE-Glycophase<sup>TM</sup> glass beads (Pierce Chemical Co.), DEAE-Sephadex<sup>TM</sup> and DEAE-Sephacel<sup>TM</sup> (Pharmacia). All DEAE-substituted matrices are weakly basic exchangers.

## METHODS

### Preparation of the Supports

Amberlite<sup>TM</sup> IR-120 and IRC-50 were converted to the ionized form with 1 M Tris base, and then washed with 10 mM Tris-HCl, pH 8.0, until the effluent reached pH 7.0 for IR-120 and pH 8.0 for IRC-50. The capacity of NTE to withstand pH changes had not been investigated at the time of these experiments, and the matrices were brought to the pH of the standard NTE assay largely as a precautionary measure. Amberlite<sup>TM</sup> IR-120 was also used as an  $\text{Al}^{3+}$  complex according to the procedure of Kothari et al. (1976). This complex probably acts as a fixed component of a chelate rather than as an ion exchanger. After conversion to the  $\text{Na}^+$  form with 1 M NaOH, the resin was washed with distilled water until the pH of the effluent was neutral. It was then equilibrated with 0.2 M  $\text{AlCl}_3$  and finally washed with 0.01 M Tris-HCl, pH 8.0, until the effluent reached pH 8.0. Amberlite<sup>TM</sup> IR-45 was converted to the  $\text{Cl}^-$  form with 1 M HCl and then washed with distilled water until the effluent was chloride free and the pH had begun to rise. The washed resin was stored in 0.01 M sodium acetate, adjusted to pH 5.0, until used for immobilization. Dowex<sup>TM</sup> 1 and 2 were equilibrated with 1 M Tris base and then washed with 0.01 M Tris-HCl, pH 8.0, until the effluent reached pH 8.0. The resins were stored in the washing buffer until used. Carboxymethyl cellulose, DEAE-cellulose and DEAE-Selectacel<sup>TM</sup> were hydrated overnight in distilled  $\text{H}_2\text{O}$ , filtered with suction, and used without further treatment. DEAE-Sephacel<sup>TM</sup> was centrifuged and decanted after hydration. DEAE-Sephacel<sup>TM</sup> was brought to pH 7.0 by the dropwise addition of 1 M NaOH and then washed twice with  $\text{H}_2\text{O}$  to remove excess base. The pH of the second wash was between 4.0 and 5.0.

### Solubilization and Immobilization

NTE was solubilized from hen or chick brain with Triton X-100 or sodium cholate essentially according to the procedures given in SECTION 9. Optimal conditions for solubilization were being worked out independently but at the same time as the immobilization experiments; thus conditions used in immobilization experiments were not always those finally established to be optimal.

The liquid phase was decanted from washed and swollen matrices in bead form, and from DEAE-Sephadex<sup>TM</sup> and -Sephacel<sup>TM</sup> after centrifugation. Other cellulose derivatives were filtered with suction. One gram of damp matrix was transferred to a 30 ml round-bottomed centrifuge tube. Generally 4 ml of soluble NTE and 4 ml of buffer or  $\text{H}_2\text{O}$  were added. Soluble NTE (7 ml), containing 75 to 100 nmol/min/ml activity, was added to the packed DEAE-Sephacel<sup>TM</sup> from 4 ml of the commercial suspension. Control tubes with NTE and buffer or  $\text{H}_2\text{O}$  but no matrix, and with matrix and buffer or  $\text{H}_2\text{O}$  but no enzyme were also prepared. The former are used to determine how much NTE was added to the matrix, corrected

for whatever inactivation might have occurred under the conditions used for immobilization. The latter show what effect the matrix has on the NTE assay.

Immobilization was allowed to proceed for 1 hr with gentle magnetic stirring in an ice bath. After overnight storage at 4°C, the matrices were sedimented, and clear supernatant removed for determination of unbound NTE. The matrices were then washed with four 8 ml portions of buffer or H<sub>2</sub>O to remove unbound enzyme. Weighed aliquots of damp matrix (from 75 to 200 mg per assay tube) or 1 ml aliquots of a suspension of DEAE-Sephadex<sup>TM</sup>, Sephacel<sup>TM</sup> and microcrystalline cellulose were used for determination of bound NTE. Exceptions to the above techniques were noted in the Results section.

#### NTE Assay

NTE activity was determined essentially as described in SECTION 3. However, when solid samples (matrix carrying immobilized NTE) were analyzed, samples were weighed into assay tubes and suspended in 1 ml of H<sub>2</sub>O or buffer. The timing of the assay was begun with the addition of inhibitors. In the standard procedure developed for liquid samples, inhibitors are put into the assay tubes first, and the timing of the assay is begun with the addition of the sample. For pipetable suspensions, such as NTE-DEAE-Sephadex<sup>TM</sup> and -Sephacel<sup>TM</sup> and microcrystalline DEAE-cellulose, the standard procedure was used.

### RESULTS AND DISCUSSION

#### Immobilization on Ion Exchangers

All five synthetic ion exchange resins tested removed NTE from solution during batch-type immobilization experiments (Table 24). The greatest binding power was exhibited by the weakly basic resin Amberlite<sup>TM</sup> IR-45. However in all cases the bound enzyme was inactive except for very small amounts of activity found on Amberlite<sup>TM</sup> IR-120-Al<sup>+3</sup> and Amberlite<sup>TM</sup> IRC-50.

All of these resins except Amberlite<sup>TM</sup> IR-45 are fully ionized and at maximum binding capacity at pH 8.0, the pH generally used for preparation and assay of NTE. In order to achieve maximum binding on Amberlite<sup>TM</sup> IR-45, it was necessary to carry out the immobilization at the lower pH. Soluble NTE was found to be stable at pH's as low as 4.0 (SECTION 9). Therefore, the disappearance of NTE during incubation with Amberlite<sup>TM</sup> IR-45 at pH 4.9 was probably not denaturation at low pH, but rather binding in an inactive form.

In some experiments the final wash after immobilization was also assayed. No appreciable amount of NTE was ever found.

TABLE 24. ATTEMPTED IMMOBILIZATION OF NTE  
ON ION EXCHANGE RESINS

| MATRIX                                                     | AM'T NTE <sup>a</sup><br>USED PER<br>G MATRIX | % NTE LOST<br>FROM LIQUID<br>PHASE DURING<br>IMMOBILIZATION | AM'T ACTIVE NTE <sup>a</sup><br>IMMOBILIZED<br>PER G MATRIX |
|------------------------------------------------------------|-----------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|
| Amberlite <sup>TM</sup><br>IR-120<br>Al <sup>3+</sup> form | 93                                            | 24.6                                                        | 0.7                                                         |
| Amberlite <sup>TM</sup><br>IR-120<br>Na <sup>+</sup> form  | 202                                           | 6.6                                                         | 0                                                           |
| Amberlite <sup>TM</sup><br>IRC-50                          | 202                                           | 16.2                                                        | 1.0                                                         |
| Amberlite <sup>TM</sup><br>IR-45                           | 109                                           | 96.3                                                        | 0                                                           |
| Dowex <sup>TM</sup> <sub>2</sub>                           | 202                                           | 37.0                                                        | 0                                                           |
| Dowex <sup>TM</sup> <sub>1</sub>                           | 202                                           | 34.0                                                        | 0                                                           |

<sup>a</sup> nmol/min.

NTE was solubilized with 1% Na cholate in 50 mM Tris, 2 mM EDTA, pH 8.0, and immobilized in the presence of 0.5% Na cholate and the same buffer for all resins except Amberlite IR-45. In this case, 1% Na cholate in 10 mM Na acetate, pH 5.9, was used to solubilize. The final pH of the soluble NTE plus resin was 4.9.



A number of attempts were made to increase the amount of NTE bound by Amberlite<sup>TM</sup> IR-50 (Table 25). Replacement of sodium cholate with the non-ionic detergent Triton X-100 doubled the percentage of enzyme bound by the resin, but did not preserve its activity. Incubation of the resin with larger amounts of soluble NTE increased the absolute amount of NTE bound, while the percentage bound dropped slightly. Elimination of buffer from the immobilization mixture did not influence binding of NTE to the resin.

Immobilization in the presence of substrate at twice the concentration used in the standard NTE assay had no effect on the amount of activity found on the resin. A similar result was obtained with 10 and 50  $\mu$ M (I<sub>50</sub> and I<sub>90</sub> concentrations) phenylbenzyl carbamate, a reversible NTE inhibitor (Johnson and Lauwerys, 1969). Reduction in the concentration of detergent by dilution and by dialysis did not increase the amount of active NTE bound.

Since these tactics failed to increase the yield of activity bound on IRC-50, immobilization was attempted on carboxymethyl cellulose (CMC), a matrix which resembles IRC-50 with respect to carboxyl substitution but differs markedly in the chemical nature of the polymer chain. DEAE-substituted cellulose was also tried since NTE bound readily to the weakly basic Amberlite<sup>TM</sup> exchanger.

The degree of binding of soluble NTE by these hydrophobic cellulosic exchangers, judged by the disappearance of enzyme from the liquid phase of the immobilization mixture, was similar to that obtained with the corresponding relatively hydrophobic resins used initially (Table 26). Most of these experiments were done with Triton solubilized enzyme and without buffer since Mitz and Schlueter (1959) report that low salt concentration favors adsorption on cellulose. The NTE bound by CMC was still totally inactivated, but a substantial amount of activity was found on DEAE-Selectacel<sup>TM</sup> (Table 26). DEAE-cellulose from Sigma Chemical Co. gave a similar result. Simultaneously reducing the initial NTE and detergent concentrations decreased the amount of activity recovered on DEAE-Selectacel<sup>TM</sup>; therefore, a high concentration of NTE is apparently more important than detergent concentration in the immobilization mixture. DEAE-substituted Glycophase<sup>TM</sup> glass beads (Pierce Chemical Co.) and microgranular DEAE-cellulose (Whatman) removed NTE from solution as readily as DEAE-Selectacel<sup>TM</sup>, but little or no activity was found on these supports.

Active NTE was also obtained in good yield on DEAE-Sephacel<sup>TM</sup> (Table 26). This preparation has the advantage of being pipetable as a suspension, eliminating the need for weighing out aliquots of NTE-loaded matrix for assay. DEAE-Sephadex<sup>TM</sup> also bound NTE quite readily with retention of activity. However, this material had a pronounced tendency to clump and to adhere to the walls of

TABLE 25. ATTEMPTED IMMOBILIZATION OF NTE ON AMBERLITE<sup>TM</sup> IRC-50  
ION EXCHANGE RESIN UNDER VARIOUS CONDITIONS

| SOLUBILIZATION<br>CONDITIONS                           | IMMOBILIZATION<br>CONDITIONS                                       | AMT. NTE<br>USED PER<br>G RESIN* | % LOSS UPON<br>INCUBATION<br>WITH RESIN | AMT. NTE<br>FOUND PER<br>G RESIN* | % NTE<br>RECOVERED<br>ON RESIN |
|--------------------------------------------------------|--------------------------------------------------------------------|----------------------------------|-----------------------------------------|-----------------------------------|--------------------------------|
| 1% Na Cholate<br>50 mM Tris pH 8                       | 0.5% Na Cholate<br>50 mM Tris pH 8                                 | 261.3                            | 28.4                                    | 4.7                               | 6.3                            |
| 0.2% Triton<br>X-100<br>50 mM Tris pH 8                | 0.1% Triton X-100<br>50 mM Tris pH 8                               | 127.3                            | 59.1                                    | 6.0                               | 8.0                            |
| 0.2% Triton<br>X-100<br>10 mM Tris pH 8                | 0.1% Triton X-100<br>10 mM Tris pH 8                               | 258.9                            | 53.2                                    | 1.3                               | 0.9                            |
| 0.2% Triton<br>X-100<br>(no buffer)                    | 0.1% Triton X-100<br>(no buffer)                                   | 333.3                            | 52.2                                    | 0.7                               | 0.4                            |
| 0.2% Triton<br>X-100<br>50 mM Tris pH 8                | 0.1% Triton X-100<br>50 mM Tris pH 8                               | 68.8                             | --                                      | 1.0                               | -                              |
| 0.2% Triton<br>X-100<br>50 mM Tris pH 8                | 0.1% Triton X-100<br>50 mM Tris pH 8<br>2.81 mM phenyl<br>valerate | 68.8                             | --                                      | 0.3                               | -                              |
| 0.2% Triton<br>X-100<br>50 mM Tris pH 8<br>(continued) | 0.1% Triton X-100<br>50 mM Tris pH 8                               | --                               | --                                      | 3.4                               | -                              |

TABLE 25 (continued)

| SOLUBILIZATION<br>CONDITIONS            | IMMOBILIZATION<br>CONDITIONS                                                  | AMT. NTE<br>USED PER<br>G RESIN* | % LOSS UPON<br>INCUBATION<br>WITH RESIN | AMT. NTE<br>FOUND PER<br>G RESIN* | % NTE<br>RECOVERED<br>ON RESIN |
|-----------------------------------------|-------------------------------------------------------------------------------|----------------------------------|-----------------------------------------|-----------------------------------|--------------------------------|
| 0.2% Triton<br>X-100<br>50 mM Tris pH 8 | 0.1% Triton X-100<br>50 mM Tris pH 8<br>10 $\mu$ M phenyl benzyl<br>carbamate | --                               | --                                      | 0.7                               | -                              |
| 0.2% Triton<br>X-100<br>50 mM Tris pH 8 | 0.1% Triton X-100<br>50 mM Tris pH 8<br>50 $\mu$ M phenyl benzyl<br>carbamate | --                               | --                                      | 1.0                               | -                              |
| 1% Na Cholate<br>50 mM Tris pH 8        | 0.25% Na Cholate<br>50 mM Tris pH 8                                           | 116.0                            | --                                      | 3.4                               | -                              |
| 1% Na Cholate<br>50 mM Tris pH 8        | 0.15% Na Cholate<br>50 mM Tris pH 8                                           | 52.4                             | --                                      | 1.7                               | -                              |
| 1% Na Cholate<br>50 mM Tris pH 8        | Dialyzed NTE<br>50 mM Tris pH 8                                               | 52.4                             | 0                                       | 1.3                               | -                              |
| 1% Na Cholate<br>50 mM Tris pH 8        | Dialyzed in the<br>presence of resin<br>50 mM Tris pH 8                       | 52.2                             | 0                                       | 2.0                               | -                              |

\*nmol/min.

TABLE 26. IMMOBILIZATION OF NTE ON CELLULOSIC AND GLASS BEAD ION EXCHANGERS

| SOLUBILIZATION<br>CONDITIONS        | IMMOBILIZATION<br>CONDITIONS       | AMT. NTE<br>USED PER <sup>a</sup><br>G MATRIX | % LOSS UPON<br>INCUBATION<br>WITH MATRIX | AMT. NTE<br>FOUND PER <sup>b</sup><br>G MATRIX | % NTE<br>RECOVERED<br>ON MATRIX |
|-------------------------------------|------------------------------------|-----------------------------------------------|------------------------------------------|------------------------------------------------|---------------------------------|
| <u>CM-CELLULOSE</u>                 |                                    |                                               |                                          |                                                |                                 |
| 1% Na Cholate<br>50 mM Tris pH 8    | 0.2% Na Cholate<br>50 mM Tris pH 8 | 16                                            | 33.7                                     | 0                                              | 0                               |
| 0.2% Triton X-100<br>(unbuffered)   | 0.2% Triton X-100<br>(unbuffered)  | 322                                           | 4.5                                      | 0                                              | 0                               |
| 0.1% Triton X-100<br>(unbuffered)   | 0.1% Triton X-100<br>(unbuffered)  | 633                                           | 8.2                                      | 0                                              | 0                               |
| <u>DEAE-SELECTACEL<sup>TM</sup></u> |                                    |                                               |                                          |                                                |                                 |
| 0.2% Triton X-100<br>(unbuffered)   | 0.2% Triton X-100<br>(unbuffered)  | 256                                           | 100.0                                    | 88.8                                           | 34.7                            |
| 0.2% Triton X-100<br>(unbuffered)   | 0.2% Triton X-100<br>(unbuffered)  | 209                                           | 100.0                                    | 47.7                                           | 22.8                            |
| 0.2% Triton X-100<br>(unbuffered)   | 0.2% Triton X-100<br>(unbuffered)  | 516                                           | 95.6                                     | 165.5                                          | 33.5                            |
| 0.2% Triton X-100<br>(unbuffered)   | 0.1% Triton X-100<br>(unbuffered)  | 184                                           | 99.5                                     | 70.8                                           | 38.6                            |
| 0.2% Triton X-100<br>(unbuffered)   | 0.05% Triton X-100<br>(unbuffered) | 66                                            | 79.1                                     | 26.2                                           | 50.0                            |
| (continued)                         |                                    |                                               |                                          |                                                |                                 |

TABLE 26 (continued)

| SOLUBILIZATION<br>CONDITIONS                    | IMMOBILIZATION<br>CONDITIONS       | AMT. NTE<br>USED PER<br>G MATRIX* | % LOSS UPON<br>INCUBATION<br>WITH MATRIX | AMT. NTE<br>FOUND PER<br>G MATRIX* | % NTE<br>RECOVERED<br>ON MATRIX |
|-------------------------------------------------|------------------------------------|-----------------------------------|------------------------------------------|------------------------------------|---------------------------------|
| <u>DEAE-CELLULOSE (Sigma)</u>                   |                                    |                                   |                                          |                                    |                                 |
| 0.2% Triton-X100<br>(unbuffered)                | 0.2% Triton X-100<br>(unbuffered)  | 209                               | 100.0                                    | 55.7                               | 24.3                            |
| <u>DEAE-CELLULOSE (MICROCRYSTALLINE)</u>        |                                    |                                   |                                          |                                    |                                 |
| 0.1% Triton X-100<br>(unbuffered)               | 0.1% Triton X-100<br>(unbuffered)  | 256                               | 99.7                                     | 1.4                                | 0.5                             |
| <u>DEAE-SEPHACEL<sup>TM</sup></u>               |                                    |                                   |                                          |                                    |                                 |
| 0.1% Triton X-100<br>(unbuffered)               | 0.1% Triton X-100<br>(unbuffered)  | 290 <sup>b</sup>                  | 96.4                                     | 199.0 <sup>b</sup>                 | 71.2                            |
| <u>DEAE-GLYCOPHASE<sup>TM</sup> GLASS BEADS</u> |                                    |                                   |                                          |                                    |                                 |
| 0.1% Triton X-100<br>(unbuffered)               | 0.05% Triton X-100<br>(unbuffered) | 777                               | 99.7                                     | 0                                  | 0                               |

<sup>a</sup> nmol/min.<sup>b</sup> per ml commercial gel suspension.



tubes used for immobilization and for assay (both plastic and glass) and also to the plastic pipet tips. Consequently, agreement between duplicate tubes in the NTE assay was so poor that a reliable determination of the amount of activity on this matrix was not possible. At this point, DEAE-Sephacel<sup>TM</sup> is the matrix of choice for the preparation of immobilized NTE.

#### Effects of Ion Exchangers on the NTE Assay

When the activity of immobilized NTE is determined, the immobilization support is present throughout the assay. These ion exchangers might be able to sequester either the enzyme reaction product (phenol) or one or more of the color reagents. If this occurs, the amount of active bound enzyme might be seriously underestimated or perhaps not detected at all.

The recovery of phenol in the presence of eleven supports used in this study was investigated in two experiments (Table 27). In the first, a known amount of phenol was incubated with each support for 12 min. The incubation mixtures were centrifuged and the liquid phase assayed. Any decrease in recovery of phenol in this experiment must be due to binding of phenol by the support. Binding seems to occur with every support tested and increases with increasing basicity for the synthetic exchangers. However, only moderate amounts (3 to 13%) of phenol were bound by the DEAE cellulose.

In the second experiment, phenol was added to each support and the assay carried out with the support present. This corresponds to the procedure used for immobilized NTE. In all cases, except three, a higher recovery was obtained than when the liquid phase was separated from the exchanger. This indicates that binding of phenol is the principal mechanism of interference, and that even "bound" phenol may be able to react with the color reagents to some extent. The DEAE celluloses and DEAE-Sephacel<sup>TM</sup> were the exceptions to this rule. However, the recovery of phenol in the presence of exchanger was only slightly lower than when the liquid phase was assayed. This effect was confirmed in a second experiment not shown.

Even though considerable interference was seen for certain resins, in no case could failure to detect NTE activity on the resin be due to interference in the assay by the matrix. However, the amount of NTE found on DEAE-substituted cellulose probably underestimates the amount of activity present by 15 to 20%.

#### Linearity of the Assay for Immobilized NTE

The relationship between NTE activity found and the amount of NTE-DEAE-Sephacel<sup>TM</sup> taken for assay is shown in Figure 36. Each of the lines was derived from a different batch of immobilized enzyme having a somewhat different NTE activity per ml gel

TABLE 27. THE EFFECTS OF THE VARIOUS ION EXCHANGERS  
USED FOR IMMOBILIZATION ON THE COLORIMETRIC DETERMINATION  
OF PHENOL

| MATRIX                                                | % RECOVERY OF<br>Ø-OH IN LIQUID<br>PHASE ONLY <sup>a</sup> | % RECOVERY OF<br>Ø-OH IN LIQUID<br>PHASE + MATRIX <sup>b</sup> |
|-------------------------------------------------------|------------------------------------------------------------|----------------------------------------------------------------|
| Amberlite <sup>TM</sup> IR-120, Al <sup>3+</sup> form | 91.1                                                       | 96.9                                                           |
| Amberlite <sup>TM</sup> IR-120, Na <sup>+</sup> form  | 89.5                                                       | 97.4                                                           |
| Amberlite <sup>TM</sup> IRC-50                        | 89.6                                                       | 96.3                                                           |
| Carboxymethyl Cellulose                               | 79.8                                                       | 99.0                                                           |
| Amberlite <sup>TM</sup> IR-45                         | 59.6                                                       | 79.8                                                           |
| DEAE-Selectacel <sup>TM</sup>                         | 87.3                                                       | 83.2                                                           |
| DEAE-Cellulose (Sigma)                                | 86.5                                                       | 81.6                                                           |
| DEAE-Sephacel <sup>TM</sup>                           | 97.8                                                       | 94.4                                                           |
| DEAE-Glycophase <sup>TM</sup> Glass Beads             | 85.7                                                       | 91.4                                                           |
| Dowex <sup>TM</sup> 2                                 | 33.9                                                       | 72.9                                                           |
| Dowex <sup>TM</sup> 1                                 | 45.2                                                       | 65.6                                                           |

<sup>a</sup> A known amount of phenol was added to the matrix; the supernatant was removed for assay.

<sup>b</sup> A known amount of phenol was added to the matrix and the mixture assayed.

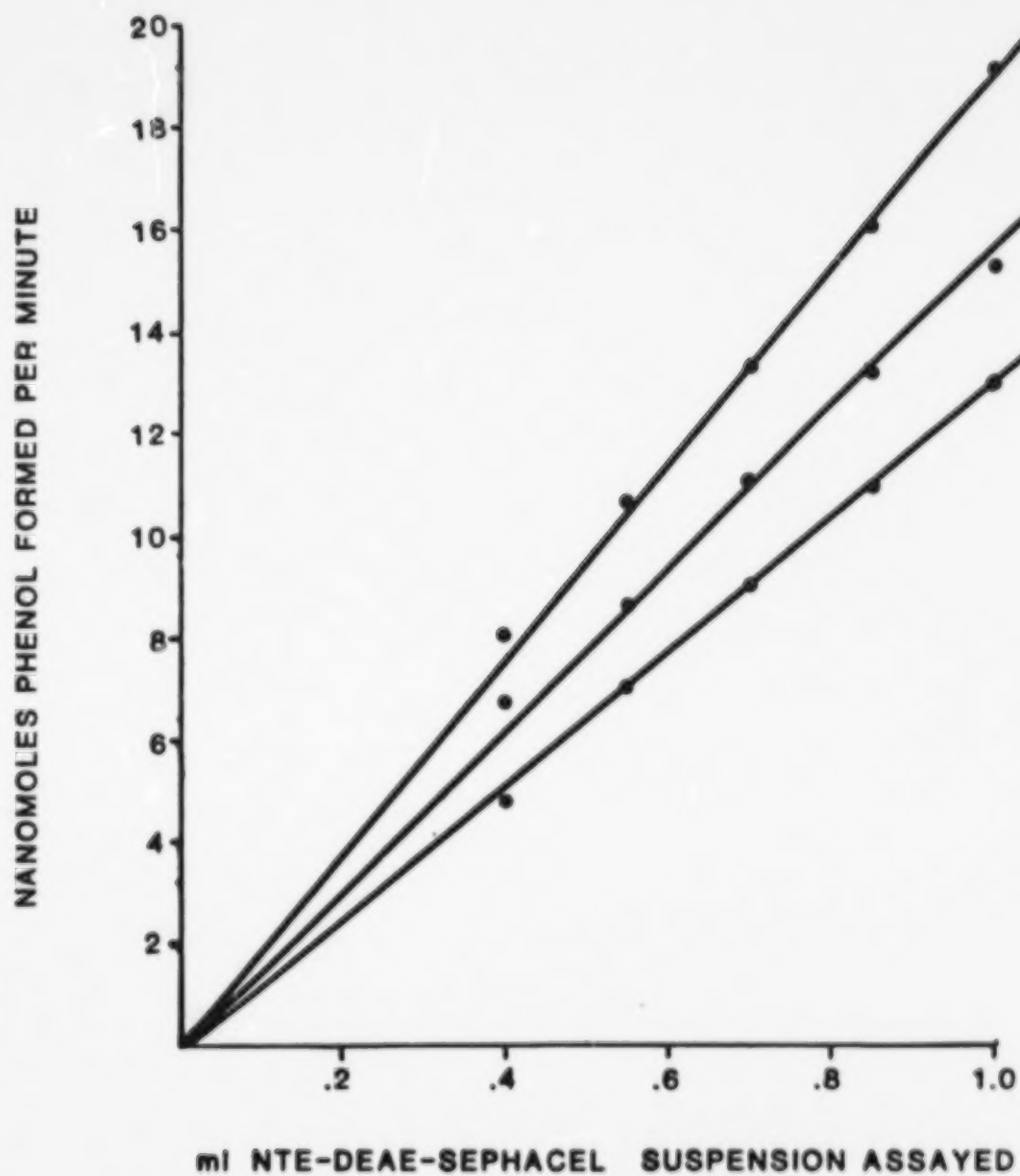


Figure 36. Relationship between the amount of NTE-DEAE-Sephacel<sup>TM</sup> assayed and NTE activity found in 3 separate experiments.

suspension. Even though this support tends to adhere to the walls of the glass tubes in which the assay is run, a linear relationship was obtained.

#### Titration Curves

Paraoxon and mipafox titration curves similar to those described in SECTIONS 5 and 9 were obtained for NTE-DEAE-Sephacel<sup>TM</sup>. The experimental protocol is described in SECTION 3. The curves are shown in Figure 37, along with the curves for NTE in whole brain homogenate for comparison. Some difference is seen in the shape of the paraoxon curve, suggesting that the immobilized paraoxon-sensitive enzymes are somewhat different in kind or behavior from those titrated in whole brain homogenate. The mipafox titration curves are quite similar for the two preparations, indicating that immobilization does not affect the reactivity of NTE. The  $PI_{50}$ 's for mipafox are  $5.10 \times 10^{-5}$  and  $5.15 \times 10^{-5}$  for native and bound NTE enzyme respectively.

#### Stability of NTE Immobilized on DEAE-Cellulose

NTE immobilized on DEAE-cellulose (Sigma), -Selectacel<sup>TM</sup> or -Sephacel<sup>TM</sup>, and stored at 4°C as a damp filter cake lost approximately 4% activity per day (Table 28). Triton solubilized NTE stored at 4°C lost 10% or less of its activity per week. Therefore, immobilization on ion exchange matrices decreased the stability of the enzyme, compared to soluble NTE kept at the same temperature. Material allowed to air dry in a desiccator at 4°C was even less stable.

Immobilized NTE stored as a suspension was less stable than when stored as a damp filter cake (Table 29). Omitting buffer from, or adding substrate to, the storage medium did not enhance stability.

Ionic binding to DEAE-substituted matrices depends on the amine group of the matrix being protonated. Consequently, high pH might dislodge enzyme from the matrix. In order to check for this effect, NTE immobilized on DEAE-Selectacel<sup>TM</sup> was stored overnight at pH's from 6.0 to 9.0. A substantial increase in stability was seen at pH 6.0 (Table 29). However, since a graded response was not observed over several pH units, the increase in stability at pH 6.0 might be related to some factor other than elution from the matrix.

Since soluble NTE was found to have the greatest short-term stability at pH 4.0 to 4.5 (Table 16), the effect of low pH on soluble NTE and NTE immobilized on DEAE-Selectacel<sup>TM</sup> over the period of a week was investigated. In this experiment, aliquots of soluble NTE made with 0.2% Triton, unbuffered, were diluted 1:1 with each of the three buffers to give a final buffer concentration of 20 mM. Tris-HCl was used at pH 8.0 and acetic acid-NaOH at pH 4.0 and 4.5. A fourth sample was diluted with H<sub>2</sub>O.

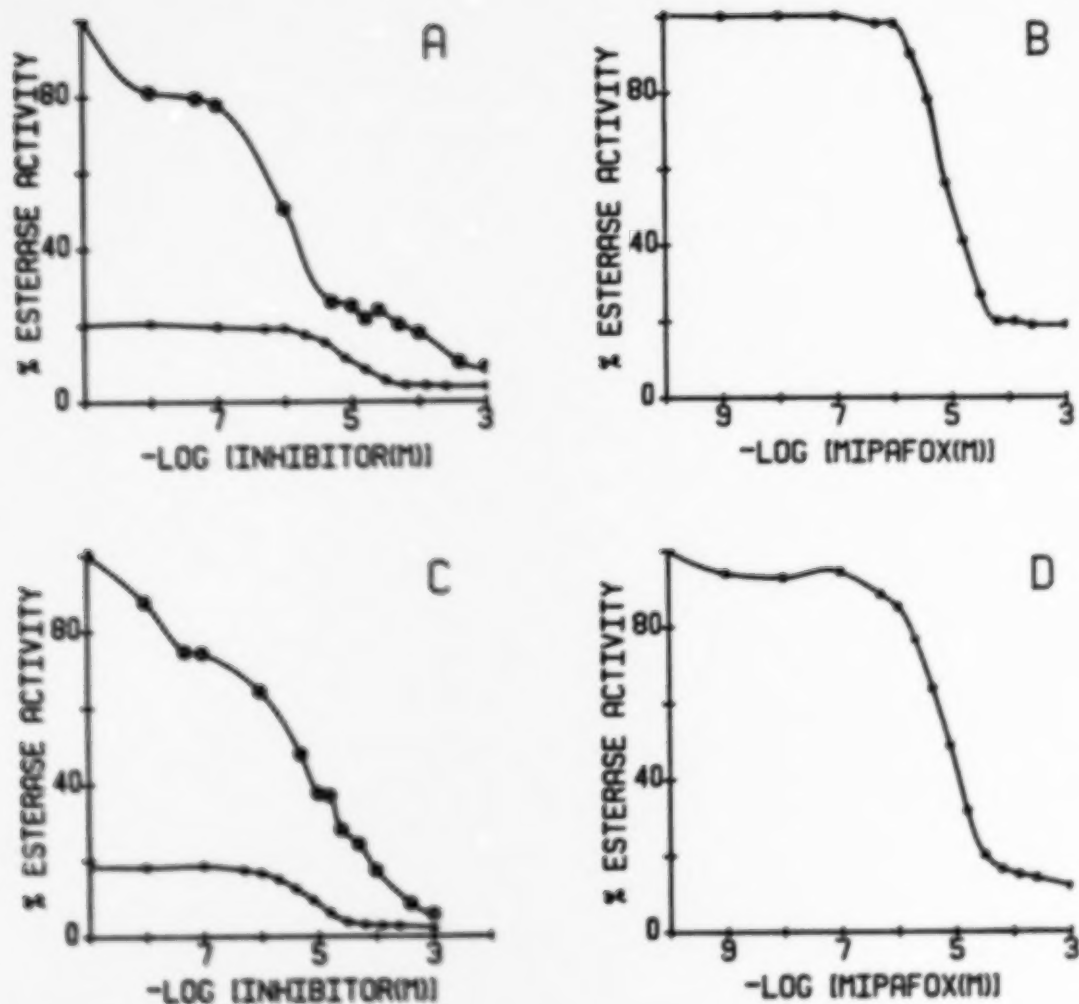


Figure 37. Titration curves for hen brain phenyl valerate activity in whole homogenate and immobilized on DEAE-Sephacel<sup>TM</sup>. A. Whole brain homogenate. B. Whole brain homogenate, mipafox titration plotted as % of activity in the presence of 100  $\mu$ M paraoxon. C. Immobilized esterase activity. D. Immobilized esterase activity, mipafox titration plotted as % of activity in the presence of 100  $\mu$ M paraoxon.  $\circ$ — $\circ$ , paraoxon;  $\bullet$ — $\bullet$ , mipafox.



TABLE 28. STABILITY OF NTE IMMOBILIZED ON DEAE-CELLULOSE

|                               | NTE ACTIVITY <sup>a</sup><br>PER G MATRIX | % CHANGE<br>PER DAY |
|-------------------------------|-------------------------------------------|---------------------|
| Initially <sup>b</sup>        | 70.8                                      |                     |
| After 5 days                  | 56.7                                      | -4.0                |
| Initially <sup>b</sup>        | 88.8                                      |                     |
| After 1 day                   | 85.6                                      | -3.6                |
| After 9 days                  | 53.7                                      | -4.4                |
| Initially <sup>b</sup>        | 55.7                                      |                     |
| After 7 days (kept damp)      | 48.4                                      | -1.9                |
| After 7 days (allowed to dry) | 25.8                                      | -7.7 <sup>c</sup>   |
| Initially <sup>d</sup>        | 47.7                                      |                     |
| After 7 days (kept damp)      | 34.9                                      | -3.8                |
| After 7 days (allowed to dry) | 15.1                                      | -9.8 <sup>c</sup>   |
| Initially <sup>e</sup>        | 15.2                                      |                     |
| After 14 days                 | 7.7                                       | -3.5                |
| Initially <sup>e</sup>        | 17.1                                      |                     |
| After 14 days                 | 5.8                                       | -4.7                |
| Initially <sup>e</sup>        | 15.5                                      |                     |
| After 14 days                 | 8.4                                       | -3.3                |

<sup>a</sup>nmol/min.<sup>b</sup>DEAE-Selectacel<sup>TM</sup>.<sup>c</sup>This figures includes any loss of activity due to drying in addition to loss of activity of the stored dry material.<sup>d</sup>DEAE-Cellulose from Sigma was used for this experiment.<sup>e</sup>NTE-DEAE-Sephacel<sup>TM</sup>. Activity is reported for 1 ml gel suspension rather than per G matrix.

TABLE 29. STABILITY OF NTE-DEAE-SELECTACEL<sup>TM</sup> IN AQUEOUS MEDIA

| TREATMENT                                 | NTE ACTIVITY <sup>a</sup><br>PER G MATRIX | %<br>CHANGE <sup>b</sup> |
|-------------------------------------------|-------------------------------------------|--------------------------|
| Kept as damp filter cake                  | 85.6                                      |                          |
| 17.5 hr in H <sub>2</sub> O               | 60.8                                      | -29.0                    |
| 17.5 hr in 50 mM Tris-2 mM EDTA<br>pH 8.0 | 81.6                                      | -15.8                    |
| 17.5 hr in 2.81 mM Phenyl Valerate        | 67.8                                      | -27.9                    |
| <hr/>                                     |                                           |                          |
| Kept as damp filter cake                  | 53.7                                      |                          |
| 16 hr in 50 mM Tris at pH 9.0             | 47.0                                      | -12.5                    |
| pH 8.0                                    | 46.3                                      | -13.8                    |
| pH 7.0                                    | 46.3                                      | -13.8                    |
| 16 hr in 50 mM Phosphate at pH 6.0        | 50.7                                      | - 5.6                    |

<sup>a</sup>nmol/min.

<sup>b</sup>Compared to the activity of the sample stored as a damp filter cake.

Aliquots of immobilized NTE were also slurried in each of the 20 mM buffers and H<sub>2</sub>O. The immobilization matrix raised the pH of the acidic buffers by 1 unit or more, so that the pH of the buffer used to make the slurry does not represent the pH at which the enzyme was stored, and has meaning only in an operational sense. However, the low ionic strength of these buffers probably favors stability of the soluble enzyme.

After storage at one week under these conditions, immobilized NTE appears to be equally stable when slurried with buffer at pH 4.0, 4.5 or in H<sub>2</sub>O, but less stable at pH 8.0 (Table 30). Even so, the preparation stored at pH 8.0 is the only immobilized NTE preparation to be more stable than the corresponding soluble NTE. In general, however, immobilization does not enhance the stability of this enzyme.

TABLE 30. THE EFFECT OF pH ON THE STABILITY OF SOLUBLE NTE AND NTE-DEAE-CELLULOSE.

| NOMINAL<br>pH    | % ACTIVITY REMAINING<br>AFTER 1 WEEK AT 4°C |                | % CHANGE PER DAY |                |
|------------------|---------------------------------------------|----------------|------------------|----------------|
|                  | SOLUBLE<br>NTE                              | IMMOB'D<br>NTE | SOLUBLE<br>NTE   | IMMOB'D<br>NTE |
| 4.0              | 77.5                                        | 63.3           | -3.2             | -5.2           |
| 4.5              | 67.1                                        | 62.3           | -4.7             | -5.4           |
| 8.0              | 35.0                                        | 56.8           | -9.3             | -6.2           |
| H <sub>2</sub> O | 83.6                                        | 65.4           | -2.3             | -4.9           |

#### Glutaraldehyde Cross-Linking of Immobilized NTE

Cross-linking with glutaraldehyde has been reported to increase the stability of microencapsulated catalase (Chang, 1971). NTE immobilized on DEAE-cellulose (Sigma) and DEAE-Selectacel™ was cross-linked according to this published method except that residual aldehyde groups were allowed to react with Tris buffer instead of NaBH<sub>4</sub>.

Two g of NTE-DEAE-cellulose weighed as the damp filter cake was slurried in 50 ml 0.1 M phosphate buffer, pH 8.0, containing 1 mM benzamidine. One-tenth ml of a 50% solution of glutaraldehyde in H<sub>2</sub>O was added and the slurry stirred for 1 hr at room temperature. Fifty ml of cold 50 mM Tris-HCl, pH 8.0, was added after 20 min, the slurry was filtered with suction, washed 3 times with 20 ml portions of cold H<sub>2</sub>O, and stored at 4°C as a damp filter cake. Cross-linking was also done at several pH's

in the presence of albumin. The cross-linked product was sometimes but not consistently more stable than the non-cross-linked material (Table 31). About two-thirds of the bound enzyme was inactivated during reaction with glutaraldehyde. Cross-linking in the presence of albumin reduced the inactivation to about 50%, but these samples were consistently less stable than the non-cross-linked material.

TABLE 31. THE EFFECT OF CROSS-LINKING WITH GLUTARALDEHYDE ON THE STABILITY OF IMMOBILIZED NTE

| pH OF<br>CROSS-<br>LINKING        | ALBUMIN | AM'T NTE <sup>a</sup><br>PER<br>G MATRIX | % LOSS OF<br>NTE DUE TO<br>CROSS-LINKING | % CHANGE IN<br>NTE ACTIVITY<br>PER DAY |
|-----------------------------------|---------|------------------------------------------|------------------------------------------|----------------------------------------|
| NTE-DEAE-Cellulose (Sigma)        |         |                                          |                                          |                                        |
| -                                 | -       | 47.7                                     | -                                        | -3.8                                   |
| 8.0                               | Absent  | 15.8                                     | 66.9                                     | +0.3                                   |
| NTE-DEAE-Selectacel <sup>TM</sup> |         |                                          |                                          |                                        |
| -                                 | -       | 55.7                                     | -                                        | -1.9                                   |
| 8.0                               | Absent  | 16.1                                     | 71.1                                     | -1.2                                   |
| -                                 | -       | 45.4                                     | -                                        | -2.9                                   |
| 8.0                               | Absent  | 15.0                                     | 67.0                                     | -4.1                                   |
| 8.0                               | Present | 19.4                                     | 57.2                                     | -6.8                                   |
| 7.5                               | Present | 19.0                                     | 58.2                                     | -5.2                                   |
| 7.0                               | Present | 25.4                                     | 44.0                                     | -8.0                                   |
| 6.5                               | Present | 23.9                                     | 47.3                                     | -8.7                                   |

<sup>a</sup> nmol/min.

NTE was solubilized and immobilized in unbuffered 0.2% Triton X-100. Immobilized NTE was stored as a damp filter cake at 4°C. Samples with dashes in some columns are control samples not subjected to the cross-linking procedure.

#### Immobilization by Hydrophobic Bonding

An attempt was made to immobilize NTE by means of hydrophobic binding to the phenoxyacetyl cellulose support Enzorb<sup>TM</sup> A. Incubation of soluble NTE with Enzorb<sup>TM</sup> A resulted in the disappearance of all NTE from the liquid phase of the incubation mixture, but no activity was found on the matrix. Attempts to elute active NTE from this support with high ionic strength buffer were unsuccessful. This matrix was not checked for binding

of phenol, which is likely to occur. This or other hydrophobic supports might be useful for purification, if not immobilization, provided that binding is not so strong as to preclude elution.

#### Immobilization by Covalent Bonding

It is generally thought that covalent attachment yields a more stable product, with respect to dissociation from the matrix than ionic binding. Three methods of covalent bond formation were used in this study: reaction with cyanogen bromide activated Sepharose<sup>TM</sup> 4-B, glutaraldehyde bonding to aminoethyl cellulose, and carbodiimide bonding to aminoethyl cellulose, carboxymethyl cellulose, and Amberlite<sup>TM</sup> IRC-50 (polymethacrylic acid).

##### Cyanogen Bromide Activated Sepharose 4-B--

This material is thought to react with primary amine groups on the enzyme (Goldstein and Manecke, 1976a). The procedure used was that supplied by Pharmacia. The dry gel was washed and swollen with 1 mM HCl. One sample of gel was partially inactivated by incubation with 0.1 M NaHCO<sub>3</sub> for 3 hr at room temperature. Complete inactivation was achieved by incubating the matrix with 1 M ethanolamine, pH 8.0, for 3 hr at room temperature. These gels were washed with coupling buffer before incubation with enzyme. Incubation with Triton solubilized NTE was carried out for 2 hr at room temperature in 0.1 M NaHCO<sub>3</sub> - 0.5 M NaCl (coupling buffer). The gel was sedimented and the liquid phase removed for determination of unbound NTE. The gel was washed once with coupling buffer and then reacted with 1 M ethanolamine, pH 8.0, for 2 hr at room temperature to inactivate any remaining bonding sites on the matrix. The gel was washed alternately with 0.1 M sodium acetate - 1 M NaCl, pH 4.0, and 0.1 M borate - 1 M NaCl, pH 8.0, three times, then washed twice with H<sub>2</sub>O to remove the salt. The pellets were resuspended in H<sub>2</sub>O for assay.

The data from these experiments is given in Table 32. Most of the NTE added to the fully active and even partially inactivated gel disappeared from the incubation medium, but very little active enzyme was found on the gel. Information from Pharmacia states that a reduction in the number of active sites on the gel reduces the number of points of attachment between the enzyme and the matrix, and sometimes results in higher levels of bound activity. However, this procedure was not beneficial for NTE. Total inactivation of the gel with ethanolamine nearly eliminated binding of NTE in either the active or inactive state.

The activity remaining on one of the samples of NTE-Sepharose<sup>TM</sup> was determined after 1 week's storage as a suspension in H<sub>2</sub>O at 4°C. Eighty-five percent of the NTE activity had been lost, indicating that this preparation is less stable than ionically bound material.



TABLE 32. IMMOBILIZATION OF NTE  
ON CNBr-ACTIVATED SEPHAROSE<sup>TM</sup> 4-B

| CONDITION OF MATRIX   | % NTE<br>DISAPPEARING | % NTE<br>RECOVERED<br>ON MATRIX | ACTIVITY<br>ON MATRIX <sup>a</sup> |
|-----------------------|-----------------------|---------------------------------|------------------------------------|
| Fully active          | 98.4                  | 3.8                             | 2.75                               |
| Fully active          | 93.8                  | 10.3                            | 2.5                                |
| Partially inactivated | 92.3                  | 12.9                            | 3.2                                |
| Fully inactivated     | 5.2                   | 0.5                             | 0.2                                |

<sup>a</sup> nmol/min NTE per ml gel suspension taken for assay.

#### Glutaraldehyde Bonding--

The bifunctional cross-linking agent glutaraldehyde has been used to immobilize enzymes by forming covalent bonds between primary amine groups on a solid support and on the enzyme molecule (Goldstein and Maeneke, 1976b). Attempts were made to immobilize NTE on aminoethyl cellulose with this reagent, using modifications of three published methods. In the first method (Robinson *et al.*, 1971), aminoethyl cellulose (AEC) was activated by incubation with 1% glutaraldehyde for 30 min in an ice bath. The mixture was centrifuged, the liquid phase decanted, and the pellet washed once with cold water. Triton solubilized NTE was incubated with the activated matrix in 50 mM phosphate buffer at pH 7.5 for 2 hr in an ice bath. The liquid phase was removed for assay, and the pellet washed with cold water before assay.

In the second method (Srere *et al.*, 1973), the matrix was activated with 4% glutaraldehyde in 0.1 M phosphate buffer, pH 6.88, for 4 hr at room temperature. The liquid phase was decanted and the pellet washed twice with H<sub>2</sub>O. The immobilization was carried out for 15 hr as described above.

In the third method (Habeeb, 1967), activation of the matrix and immobilization of the enzyme were carried out simultaneously. The reaction mixture consisted of matrix, 50 mM phosphate buffer, pH 6.0, 2% glutaraldehyde, and Triton-solubilized enzyme. The liquid phase was removed after an incubation of 2 hr at room temperature. The pellet was washed twice with H<sub>2</sub>O before assay.

Soluble enzyme was also exposed to the matrix in the absence of glutaraldehyde. The stability of the immobilized NTE after 7 days at 4°C was also determined. The most activity was found on the matrix which had been activated for 30 min and then exposed to NTE (Table 33). Almost as much activity was found on the matrix not treated with glutaraldehyde at all. This NTE was presumably ionically bound. All of these samples of immobilized NTE

were less stable than NTE bound to DEAE-cellulose, or Triton solubilized NTE stored at 4°C.

TABLE 33. IMMOBILIZATION OF NTE BY COVALENT BONDING TO AMINOETHYL CELLULOSE WITH GLUTARALDEHYDE

| BINDING CONDITIONS               |        |             | % NTE LOST<br>DURING<br>BINDING | NMOL/MIN NTE<br>PER ML<br>MATRIX SUSP. | % ACT.<br>AFTER<br>7 DAYS<br>AT 4°C <sup>a</sup> |
|----------------------------------|--------|-------------|---------------------------------|----------------------------------------|--------------------------------------------------|
| GLUTARALDEHYDE<br>CONC.          | TIME   | NTE<br>TIME |                                 |                                        |                                                  |
| 1%                               | 30 min | 2 hr        | --                              | 9.53                                   | 18                                               |
| 4%                               | 4 hr   | 15 hr       | 58                              | 5.30                                   | 17                                               |
| 2% + NTE, 2 hr<br>simultaneously |        |             | 96                              | 2.22                                   | 40                                               |
| 0%                               | -      | 15 hr       | 34                              | 9.17                                   | 10                                               |

<sup>a</sup>Soluble NTE retained 95-100% of its activity over 7 days storage.

#### Carbodiimide Bonding--

Carboxyl or amine groups on proteins can be linked to amine or carboxyl groups on supports respectively using water soluble carbodiimides. An attempt was made to bond NTE to aminoethyl cellulose using 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CMCDI) according to the method of Line et al. (1971). The matrix was exposed to CMCDI (0.4%) and NTE simultaneously in 25 mM acetate buffer, pH 4.0 and 4.5, and in H<sub>2</sub>O. The immobilization mixture was stirred for 1 hr at room temperature and then overnight at 4°C. Nearly all the NTE disappeared from the liquid phase of the immobilization mixture, but very little activity was found on the matrix (Table 34). Active NTE was bound to the matrix ionically, suggesting that the carbodiimide was responsible for inactivating the enzyme. Since so little covalently bound enzyme was found on the matrix, a 7-day stability study was not done.

TABLE 34. COVALENT BONDING OF NTE  
TO AMINOETHYL CELLULOSE BY CARBODIIMIDE

| <u>pH OF IMMOB. MIXTURE</u><br><u>INITIAL</u> | <u>FINAL</u> | <u>CMCDI</u><br><u>PRESENT?</u> | <u>% NTE LOST</u><br><u>DURING IMMOB.</u> | <u>AM'T NTE FOUND</u><br><u>PER ML MATRIX</u><br><u>SUSPENSION*</u> |
|-----------------------------------------------|--------------|---------------------------------|-------------------------------------------|---------------------------------------------------------------------|
| 4.00                                          | 4.33         | Yes                             | 98.5                                      | 1.28                                                                |
| 4.50                                          | 4.74         | Yes                             | 98.0                                      | 1.07                                                                |
| (H <sub>2</sub> O)                            | 7.01         | Yes                             | 97.3                                      | 0.07                                                                |
| (H <sub>2</sub> O)                            | 8.23         | No                              | 98.0                                      | 5.98                                                                |

\*nmol/min.

CMCDI was also used to link carboxyl groups on Amberlite<sup>TM</sup> IRC-50 and carboxy-methyl cellulose to amine groups on the enzyme. This approach has the advantage that the matrix carboxyl groups can be activated to the O-acylisourea derivative and the CMCDI washed out before the enzyme is added. The procedure used was essentially that of Weetal (1976). Each matrix was activated at pH 4.0 (50 mM acetate buffer) and pH 10.0 (0.1 M carbonate buffer) for 2 hr at room temperature. The carbodiimide was washed out with H<sub>2</sub>O, and soluble NTE added in 50 mM acetate, pH 4.0. These mixtures were stirred at room temperature for 2 hr. The liquid phase was saved for assay, and the pellets washed with H<sub>2</sub>O, 0.1 M acetate, pH 4.0 + 1 M NaCl, and H<sub>2</sub>O again before storage at 4°C. Although nearly all of the NTE disappeared from the immobilization mixture, very little active enzyme was found on the matrix (Table 35).

TABLE 35. ATTEMPTED COVALENT BONDING OF NTE  
TO CARBOXYL BEARING SUPPORTS WITH CARBODIIMIDE

| <u>MATRIX</u> | <u>ACTIVATION</u><br><u>pH</u> | <u>IMMOB.</u><br><u>pH</u> | <u>% NTE LOST</u><br><u>DURING IMMOB.</u> | <u>AM'T NTE BOUND</u><br><u>PER G MATRIX*</u> |
|---------------|--------------------------------|----------------------------|-------------------------------------------|-----------------------------------------------|
| IRC-50        | 4.0                            | 4.0                        | 100.0                                     | 0.15                                          |
| IRC-50        | 10.0                           | 4.0                        | 98.3                                      | 0.50                                          |
| CMC           | 4.0                            | 4.0                        | 93.5                                      | 0                                             |
| CMC           | 10.0                           | 4.0                        | 94.5                                      | 0.15                                          |

\*nmol/min.

### Summary

In a discussion of immobilization as a means of increasing enzyme stability, Klibanov (1979) states that while the technique is often successful, it is not always so. Moreover, there is no theoretical basis for an assumption that it would be. In this study NTE was immobilized with retention of catalytic activity by both covalent and ionic bonding. However, neither of these preparations equaled the stability of the free enzyme stored under comparable conditions.

Both forms of immobilized NTE were obtained with sufficient activity on the matrix to be useful as an enzyme preparation. The stability of the ionically bound form is adequate for storage over about one week. This form reacts normally in the NTE assay and retains the inhibitor characteristics of the native enzyme.



## SECTION 13

### ELECTROPHORETIC SEPARATION OF NTE ON POLYACRYLAMIDE GELS

Gel electrophoretic systems have been developed for studying NTE in both native and phosphorylated forms. Triton solubilized enzyme was applied to gels in which Triton had been included after the method of Dewald (1974). This procedure is thought to minimize disruption of the protein-detergent micelles into which Triton X-100 had been incorporated and in which activity is retained. Following electrophoresis, gels were incubated with phenyl valerate, and stained with Fast Black K which reacts specifically with phenol (Stern and Lewis, 1962). These experiments did not yield identifiable bands corresponding to NTE. Even if NTE had entered the gel and retained its activity during electrophoresis, it is possible that the amount of phenol produced during staining was insufficient for visualization in the presence of spontaneous hydrolysis of the substrate. Lowering the pH of the staining mixture to reduce spontaneous hydrolysis might overcome this problem since the pH-activity range for NTE is known to be broad (SECTION 9).

SDS-polyacrylamide vertical slab gel electrophoresis (SDS-PAGE) has been developed as a means of studying phosphorylated forms of neurotoxic esterase. This technique increases the likelihood of penetration of the enzyme or its subunits into the gel. Using the discontinuous buffer system of Laemmli (1970), enhanced resolution of protein subunits from Triton solubilized hen brain microsomes has been obtained. Phosphorylated NTE will be identified by radioactive labeling with [ $^{14}\text{C}$ ]-DFP. No radioactive labeling experiments have been performed as of yet.

This electrophoretic technique is also applicable to protein kinase studies with NTE. Protein kinase assays have been worked out in collaboration with Dr. T. Ueda in the University's Mental Health Research Institute. We have successfully demonstrated endogenous phosphorylation of chick synaptic membrane fractions by  $\gamma$ -[ $^{32}\text{P}$ ]-ATP by separating the phosphorylated protein on SDS-PAGE followed by autoradiography and protein staining with Coomassie Blue. These experiments provide the basis for investigating the possibility that NTE may be phosphorylated endogenously, and that OP delayed axonopathy is a result of the disruption of this process.



## REFERENCES

1. Abou-Donia, M.B., and Preissig, S.H. (1976). Delayed neurotoxicity of leptophos: Toxic effects on the nervous system of hens. Toxicol. Appl. Pharmacol. 35, 269-282.
2. Aldridge, W.N. (1953). The differentiation of true and pseudo cholinesterase by organophosphorus compounds. Biochem. J. 53, 62-67.
3. Anonymous (1975). "Ficoll-Paque: For in vitro isolation of lymphocytes. Pharmacia Fine Chemicals, Inc.
4. Bergman, F. (1958). The structure of the active surface of cholinesterases and the mechanisms of their catalytic action in ester hydrolysis. Adv. Catalysis 10, 130-164.
5. Bergman, F., Rimons, S., and Segal, R. (1958). Effects of pH on the activity of eel esterase towards different substrates. Biochem. J. 68, 493-499.
6. Bøyum, A. (1968). Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 21, 9-109.
7. Brestkin, A.P. and Rozengart, E.V. (1965). Cholinesterase catalysis. Nature 205, 388-389.
8. Bruice, T.C. and Gaston, L.C. (1956). The catalysis of the hydrolysis of p-nitrophenyl acetate by imidazole and its derivatives. Arch. Biochem. Biophys. 63, 484-486.
9. Chang, T.M.S. (1971). Stabilization of enzymes by microencapsulation with a concentrated protein solution or by microencapsulation followed by cross-linking with glutaraldehyde. Biochem. Biophys. Res. Commun. 44, 1531-1536.
10. Coleman, R. (1973). Membrane-bound enzymes and membrane ultrastructure. Biochim. Biophys. Acta 300, 1-30.
11. Davis, C.S., Barth, M.L., Dudek, B.R., and Richardson, R.J. (1980). Inhibitor characteristics of native, solubilized, and lipid-reconstituted neurotoxic esterase. Proceedings of the Second International Congress on Toxicology. Brussels, Belgium [in press].

12. Davis, C.S. and Richardson, R.J. (1980a). Organophosphorus compounds. In Experimental and Clinical Neurotoxicology. (P. Spencer and H. Schaumburg, eds.) Williams and Wilkins, Baltimore. pp. 527-544.
13. Davis, C.S. and Richardson, R.J. (1980b). Solubilization of neurotoxic esterase. Abstracts of the 19th Annual Meeting of the Society of Toxicology, Washington, DC, March 9-13, 1980.
14. DeCandole, C.A., Douglas, W.W., Evans, C.L., Holmes, R., Spencer, K.E.V., Tarrance, R.W., and Wilson, K.M. (1953). The failure of respiration in death by acetylcholinesterase poisoning. Brit. J. Pharmacol. 8, 466-475.
15. Dewald, B., Dulaney, J.T., and Touster, O. (1974). Solubilization and polyacrylamide gel electrophoresis of membrane enzymes with detergents. Methods in Enzymology. 32, 82-91.
16. Ellman, G.L., Courtney, D., Andres, V., Jr., and Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharm. 7, 88-95.
17. Gauthier, G.F., Lowey, S., and Hobbs, A.W. (1978). Fast and slow myosin in developing muscle fibers. Nature 274, 25-29.
18. Goldstein, L. and Manecke, G. (1976a). The chemistry of enzyme immobilization. Appl. Biochem. Bioeng. 1, 23-110.
19. Goldstein, L. and Manecke, G. (1976b). The chemistry of enzyme immobilization. Appl. Biochem. Bioeng. 1, 71.
20. Habeeb, A.F.S.A. (1967). Preparation of enzymatically active, water-insoluble derivatives of trypsin. Arch. Biochem. Biophys. 119, 264-268.
21. Hess, A.J. (1961). Structural differences of fast and slow extrafusar muscle fibers and their nerve endings in chickens. J. Physiol. (London) 157, 221-231.
22. Johnson, M.K. (1969a). The delayed neurotoxic effect of some organophosphorus compounds: identification of the phosphorylation site as an esterase. Biochem. J. 114, 711-717.
23. Johnson, M.K. (1969b). A phosphorylation site in brain and the delayed neurotoxic effect of some organophosphorus compounds. Biochem. J. 111, 487-495.

24. Johnson, M.K. (1974). The primary biochemical lesion leading to the delayed neurotoxic effects of some organophosphorus esters. J. Neurochem. 23, 785-789.
25. Johnson, M.K. (1975). The delayed neuropathy caused by some organophosphorus esters: mechanism and challenge. Crit. Revs. Toxicol. 3, 289-316.
26. Johnson, M.K. (1977). Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. Arch. Toxicol. 37, 113-115.
27. Johnson, M.K., and Lauwerys, R. (1969). Protection by some carbamates against the delayed neurotoxic effects of di-isopropylphosphorofluoridate. Nature 222, 1066-1067.
28. Klibanov, A.M. (1979). Enzyme stabilization by immobilization. Analyt. Biochem. 93, 1-25.
29. Kothari, R.M. Grafius, M.A., and Millar, D.B. (1976). A simple and rapid chromatographic method for the immobilization of acetylcholinesterase from electric eel. Analyt. Biochem. 71, 273-280.
30. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T<sub>4</sub>. Nature 227, 680-685.
31. Line, W.F., Kwong, A., and Weetall, H.H. (1971). Pepsin insolubilized by covalent attachment to glass: Preparation and characterization. Biochim. Biophys. Acta 242, 194-202.
32. Lotti, M. and Johnson, M.K. (1978). Neurotoxicity of organophosphorus pesticides: Predictions can be based on in vitro studies with hen and human enzymes. Arch. Toxicol. 41, 215-221.
33. Maizel, J.V., Jr. (1971). In: Methods in Virology. V. (Maramorosch, K. and Koprowski, H., eds). p. 203.
34. Mendoza, C.E. (1976). Toxicity and effects of malathion on esterases of suckling albino rats. Toxicol. Appl. Pharmacol. 35, 229-238.
35. Mitz, M.A. and Schlueter, R.J. (1959). Isolation of proteolytic enzymes from solution as dry stable derivatives of cellulosic ion exchangers. J.A.C.S. 81, 4024-4028.
36. Newcomer, W.S. (1958). Physiologic factors which influence acidophia induced by stress in the chicken. Am. J. Physiol. 194, 251-254.

37. Ohkawa, H., Mikami, N., Okuno, Y., and Miyamoto, J. (1977). Stereospecificity in toxicity of the optical isomers of EPN. Bull. Environ. Contam. Toxicol. 18, 534-540.
38. Poulsen, E. and Aldridge, W.N. (1964). Studies on esterases in the chicken central nervous system. Biochem. J. 19, 182-189.
39. Richardson, R.J., Davis, C.S., and Johnson, M.K. (1979). Subcellular distribution of marker enzymes and of neurotoxic esterase in adult hen brain. J. Neurochem. 32, 607-615.
40. Robinson, P.J., Dunnill, P., and Lilly, M.D. (1971). Porous glass as a solid support for immobilization or affinity chromatography of enzymes. Biochim. Biophys. Acta 242, 659-661.
41. Srere, P.A., Mattiasson, B., and Mosbach, K. (1973). An immobilized three-enzyme system: A model for microenvironmental compartmentation in mitochondria. Proc. Nat. Acad. Sci. USA 70, 2534-2538.
42. Stern, J. and Lewis, W.H.P. (1962). Serum esterase in mongolism. J. Mental Def. Res. 6, 13-21.
43. Waterman, A.J. (1971). Chordate Structure and Function. The Macmillan Co., New York.
44. Weetall, H. (1976). Covalent coupling methods for inorganic support materials. In: Methods in Enzymology. (Mosbach, K. ed.) Academic Press, pp. 144-145.
45. Whittaker, V.P. and Barker, L.A. (1972). The subcellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. In Methods of Neurochemistry, vol. 2 (Fried, R., ed.) Marcel Dekker, New York, pp. 1-52.
46. Wolford, J.H. and Ringer, R.K. (1962). Adrenal weight, adrenal ascorbic acid, adrenal cholesterol and differential leucocyte counts as physiological indicators of "stressor" agents in laying hens. Poultry Sci. 41, 1521-1529.
47. Zaborsky, O.R. (1973). Immobilized Enzymes. CRC Press, Cleveland.